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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/664,678	09/12/2003	Walter Schubert	S&H-010DX	4058

7590 04/24/2006

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EXAMINER	
CROWDER, CHUN	
ART UNIT	**PAPER NUMBER**
1644	

DATE MAILED: 04/24/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/664,678	SCHUBERT, WALTER	
	Examiner	Art Unit	
	Chun Crowder	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *08 February 2006*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-7* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-7* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

1. Applicant's amendment, filed 02/08/2006, is acknowledged.

Claims 1-7 are pending and under consideration.

2. This Office Action will be in response to applicant's arguments, filed 02/08/2006.

The rejection of record can be found in the previous Office Action, mailed 08/08/2005.

The text of those sections of Title 35 U.S.C. not included in this Action can be found in the prior Action.

3. Claims 1-7 stand rejected under **35 U.S.C. 112, first paragraph** because the specification, while being enabling for a method of blocking the cytotoxic activity of Fcγ RIII-positive ALS specific cells in a patient with amyotrophic lateral sclerosis using soluble FcγRIII, does not reasonably provide enablement for the full breath of soluble Fcγ receptors and a method of treating a patient with ALS. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the claimed invention commensurate in scope with these claims.

Applicant's arguments in conjunction with the Schubert Declaration under 37 C.F.R. 1.132, filed 02/08/2006, have been fully considered but have not been found convincing for the following reasons:

1) Applicant asserts that there are clear statements in the specification to enable how to make and use the full breath of the claimed Fcγ receptors.

Contrary to applicant's assertion, the instant disclosure does not enable skilled artisan to make and use all classes of soluble Fcγ receptors.

Example 1 on page 8 discloses "soluble Fcγ receptor preparations 50 KDa, from E. coli". This does not appear to be an enabling disclosure to a skilled artisan. The state of art recognizes that the making of soluble receptors can be unpredictable. For example, Paetz et al. (Biochemical and Biophysical Research Communications. 2005, 338:1811-1817) teach that the production of a functional, soluble form of the FcγRI receptor has been troublesome and the yields have been disappointingly low.

Further, it is not clear how *in vitro* studies reasonably predicts efficacy of the *in vivo* treatment of ALS. Teilaud et al. (Blood 1993. 82;10:3081-3090) teach that the biologic functions of soluble Fcγ receptors are largely hypothetical (see entire document, particularly pages 3081-3082).

Therefore, the specification does not reasonably provide enablement for the full breath of soluble Fcγ receptors and a method of treating a patient with ALS.

2) Applicant asserts that the therapeutic strategy of soluble Fcγ receptors is to bind the gamma region of the IgG1 and IgG3; and the statement of "subsequently 150 mg/weight kg daily, over a period of 5 days" represents the therapeutic regimen of treating amyotrophic lateral sclerosis (ALS). Further, applicant argues that based on the *in vitro* studies disclosed on pages 5-6 of the instant specification, the efficacy of the claimed method of treating was predicted.

In contrast to applicant's assertion, it is not clear whether Example 1 and the *in vitro* studies are reasonably predicative of the *in vivo* efficacy of treating ALS.

Zhang et al. (Journal of Neuroimmunology. 2005. 159:215-224) show that serum IgG levels in patients with ALS are significantly lower than normal control (see entire document, particularly page 219 and Table 2). Zhang et al. further teach that the CD16 expression on monocytes of patients with ALS is independent of severity of the disease (e.g. see pages 219-220).

Further, it is not clear how *in vitro* studies reasonably predicts efficacy of the *in vivo* treatment of ALS as addressed above in Section 1.

Furthermore, Galon et al. (The Journal of Immunology. 1996. 157:1184-1192) show that in addition to IgG, soluble CD16 (sCD16) binds various receptors including complement receptor type 3 (CR3). Moreover, interaction between sCD16 with CR3 induces the production of pro-inflammatory cytokines by monocytes and polymorphonuclear cells.

Therefore, it is unpredictable as to whether blocking *in vitro* cytotoxicity would be predicative of the efficacy of *in vivo* treatment given that sCD16 interacts with several cellular ligands and can exhibit diverse roles *in vivo*.

3) Applicant suggests that based on the subject matter, the *in vitro* studies cited and the other research results disclosed in the specification, the operativeness of the invention is reasonably predictable at the time the invention was made.

Applicant's arguments and the examiner's rebuttal, which are reiterated herein, are essentially the same as before and addressed above in Sections 1 and 2.

Further, applicant argues that the teachings of Dalaka et al. and Rudnicki et al. have no logical bearing. However, applicant does not provide other evidentiary support in the arguments.

The arguments of counsel cannot take the place of evidence in the record. In re Schulze, 145 USPQ 716, 718 (CCPA 1965). See MPEP 716.01(c).

Applicant argues that Rudnicki et al. only reduces serum IgG to 876 mg/dl in a case report of one patient.

Applicant is invited to review Clark et al. cited on page 15 and 17 of applicant's own remark. Clark et al. show that two clinical trials using plasmapheresis to remove immune components have indicated no benefit in treating ALS (see page 189 and 194 and the attachment of this Office Action).

Further, applicant argues that the teachings of Dalaka et al. of high-dose intravenous immunoglobulin (IVIG) would be expected to worsen the patients' condition because increasing IgG level in blood would activate the ALS-disease-specific immune cells.

In contrast to applicant's assertion, there is insufficient evidence that IVIG would activate the ALS-disease-specific immune cells. In fact, Samuelsson et al. (Science. 2001. 291:484-486) show that IVIG mediates anti-inflammatory activity through the inhibitory Fc receptor (see entire document, particularly page 486). In addition, Clark et al. show that the specific choice of intravenous immunoglobulin was based on both immunoglobulin's success in treating various autoimmune neuromuscular diseases and on specific findings suggesting an autoimmune component in ALS that might respond positively to immunoglobulin therapy, however, IVIG has not shown clinical benefit in treating ALS.

In addition, Applicant's Remark on page 11 states that "it is logical and reasonable to predict, solely from the inventor's research results disclosed in the specification, that any substance introduced to the serum that would selectively bind the constant gamma chain of immunoglobulin in the serum ..... would thereby selectively deprive the FcγRIII receptors on the ALS-disease-specific immune cells from binding with said immunoglobulin". However, substance such as IVIG clearly has not shown to be clinically beneficial to ALS patients.

Thus the state of the art indicated that there were potentially multiple disease mechanisms; that the role of the immune system in ALS, if any, was not clear; and that immune-based treatments were not effective.

4) Applicant asserts post-filing date biomedical research continues to support predictability of operativeness of the claimed invention of treating ALS by administering soluble Fcγ receptors.

Applicant's arguments are not found persuasive.

The Examiner has previously provided additional evidence that treatment of ALS is highly unpredictable. In addition, Clark et al. teach that clinical trial of immunosuppression in ALS using total lymphoid irradiation show no benefit although lymphoid irradiation successfully suppress cellular and humoral immune function.

Moreover, Clark et al. show that with no known cause and only one treatment of minimal efficacy (riluzole), correlating possible biochemical signs of disease progression or regression to clinical observation is difficult.

Therefore, in the absence of working examples that are reasonably predictive that the use of soluble any Fcγ receptors would function in vivo as treatment for ALS, the experimentation left to one skilled in the art to practice the claimed invention is unnecessarily, and improperly, extensive and undue.

4. Claims 1-7 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 6,649,165. Although the conflicting claims are not identical, they are not patentably distinct from each other because claims 1-7 of the '165 patent recite a method of blocking cytotoxic activity of FcγRIII positive immune cells in a ALS patient by infusing or injecting effective amount of IgG1/IgG3 binding soluble FcγRIII. The invention of the '165 patent encompasses one mechanism of treating a patient with ALS; it renders the claims 1-7 in the instant application obvious.

The rejection on the basis of double patenting will be maintained until such a time that a terminal disclaimer in compliance with 37 C.F.R. 1.321(c) has been timely filed.

5. *No claim is allowed.*

6. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Chun Crowder whose telephone number is (571) 272-8142. The examiner can normally be reached Monday through Friday from 8:30 am to 5:00 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on (571) 272-0841. The fax number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Chun Crowder, Ph.D.
Patent Examiner
April 6, 2006

PHILLIP GAMBEL, PH.D
PRIMARY EXAMINER
1600
4/10/06

Attachments: Sections from Clark et al. "Amyotrophic Lateral Sclerosis: A report on the state of research into the cause, cure, and prevention of ALS" June 2005. Clark et al. Prepared for the Dept. of Public Health, State of Massachusetts by the ALS Therapy Development Foundation. http://www.als.net/docs/ALSReport.pdf

Clinical Trials in Amyotrophic Lateral Sclerosis, (Clark et al. Pages 2-37).

Current Research Topics in ALS (pages 3-42)

Clark et al. (pages 189, 194, 239, and 245)

Clark et. al.

Clinical Trials in Amyotrophic Lateral Sclerosis

An Historical Review & Report

Amyotrophic lateral sclerosis (ALS) is an idiopathic neurodegenerative disorder for which there is no known cure and no treatment capable of dramatically slowing or arresting progression. This lack of an effective treatment, and the relatively low prevalence of ALS (an estimated 70,000 patients worldwide) has led to the perception among lay people and non-specialists that clinical investigations in ALS are still in their infancy and occur only rarely.

However, as most ALS researchers are well aware, ALS has a long and at times rich tradition of clinical investigations compared to other diseases that affect similar numbers of people. The first organized clinical trials in ALS were initiated around the time Lou Gehrig's diagnosis with ALS (1939), although this was a coincidence and not the result of Gehrig's diagnosis as has often been assumed.[1] These trials were obviously quite different from contemporary clinical trials - most were unblinded, uncontrolled, followed no definite dosing schedule, and retained many of the features of the classic case study approach to reporting therapeutic efficacy that had been used throughout the 19th and early 20th century. Initial results were reported enthusiastically in the popular press as "cures" for ALS, and it was only upon later reflection in scientific journals that researchers began to realize both the variety of neurological disorders that can mimic ALS and the inter- and intra-individual variation in disease progression.[2]

Excitement over the initiation of the first organized clinical investigations in ALS was quickly dampened by the discovery that certain patients could have transient periods of improvement and that their rate of progression could spontaneously level off – mimicking a possible therapeutic effect if these phenomena coincided with patients' entry into a 'trial.'[3] The failure of mid-century

clinical trial designs to account for this variation in disease progression was so powerful that it would be more than two decades before researchers conducted another prospective, multi-patient clinical investigation in ALS.[4]

The clinical trial landscape in ALS has understandably undergone significant changes since this early period of tentative experimentation and trial design frustration. Although there is still no treatment capable of reversing or arresting the progression of ALS, the clinical trial infrastructure necessary to demonstrate the efficacy of possible treatments has become increasingly more sophisticated in recent years, both as a result of changes in general principles of clinical trial design and conduct, and as a result of an improved understanding of the biology and epidemiology of ALS. Researchers in ALS now have an array of methods for measuring disease progression, structuring efficacy trials to maximize statistical power, balancing the desire to provide treatment to all patients with the need for placebo controls, stratifying patient populations to reduce variability, and calculating and evaluating dosing - all of which have been analyzed and validated through a series of conferences aimed at developing a consensus on clinical trial design in ALS. In addition, the growth in animal and *in vitro* models of ALS and growing attention to laboratory screening of large numbers of therapeutic candidates has provided clinicians with exponentially increasing array of possible treatments for clinical investigation.

These trends have led to considerable increases in the number of clinical trials conducted. Between 1964 and 2004, the results of a total of 134 clinical trials were published in major journals, but the majority of these trials were published in the final 10 years of this period.[5] Between 1964 and 1980 an average of one trial was published each year; by the late 1990s this number had increased to more than 7 trials each year. While these numbers are small, they are significant for the size of the patient population. By comparison, during the same time periods the average number of published clinical trials in Huntington's disease (which has a similar prevalence to

ALS) was 0.5 per year and 2 per year, respectively.[6]

In addition to increases in the number of published trials, there has been one therapeutic success in treating ALS: riluzole, a drug which inhibits pre-synaptic glutamate release. Riluzole slows, rather than stops, progression and has only a very small impact on the course of the disease. By conservative estimates, twelve to eighteen months of treatment with riluzole extends patients' post-diagnosis survival by an average of only 2 to 3 months.[7]

Nevertheless, the success in demonstrating a statistically significant survival advantage after treatment with riluzole has had a number of positive effects on ALS research. Riluzole offered the first clinical evidence that contemporary therapeutic approaches may be able to gain a foothold in slowing or arresting the progression of the disease. Riluzole's modest clinical success, FDA approval for marketing by Aventis under the trade name Rilutek®, and potential for treating a range of related neurodegenerative disorders with larger patient populations have also increased pharmaceutical and biotechnology interest in ALS as a possible therapeutic target for their investigational drug candidates. This has resulted in both new levels of academic-industry collaboration at the pre-clinical level and increased corporate involvement in clinical trials. These collaborations may ultimately reduce the time it takes for promising investigational treatments to reach ALS patients.

Overall, these changes have created a promising climate for clinical research into ALS. Corporate collaboration and expanded pre-clinical investigational techniques will likely continue to increase the range of potential candidates for clinical investigation and will provide a solid experimental basis for investigation. Increasingly sophisticated trial design will reduce the number of patients needed to demonstrate efficacy, meaning that more trials can be conducted simultaneously with the limited patient population available. If treatments are not clinically effective, the chances are higher than ever that researchers will discover this early in the course of the trial and will be able to halt the trial rather than submitting

patients to months or years of futile treatment. Although clinical trials in ALS have unfortunately not yet identified treatments capable of reversing, arresting or dramatically slowing disease progression, these clinical investigations have nevertheless helped refine trial design so that effects observed in the future will be much more likely to be 'real' rather than the result of random (but not therapeutically significant) differences between treatment and control populations.

This report traces the history of clinical trials in ALS over the past forty years, focusing on shifts in trial design and conduct that helped shape contemporary principles of trial design, tracing broad trends in the types of treatments investigated and the rationale for doing so, and assessing the implications of these trends for shaping contemporary research policy on ALS.[8] The review is organized into four separate sections and a series of Appendices. Section 1 summarizes major issues in clinical trial design in ALS, and what – if any – consensus has been reached in the research community on the appropriate resolution of these issues. This section uses these issues to sketch an outline of the ideal clinical trial in ALS as it is understood by contemporary researchers, both in terms of specific prospective design principles and in terms of envisioned outcomes. Sections 2 and 3 summarize major shifts in the study designs, patient populations, and investigational treatments used in clinical trials over the last forty years, focusing on tracing broad trends rather than addressing individual studies. These trends can help illuminate the extent to which actual clinical trial practice has diverged from or approached consensus guidelines on trial design.

Section 4 of this report consists of a series of case studies of investigational therapies on which more than one trial has been conducted and on which at least one major efficacy trial has been conducted.[9] The purpose of these case studies is to provide an overview of the history of clinical investigations of each treatment, with brief comments on the positives or negatives of study design, and to provide references (when available) to any Cochrane or other systematic reviews that have been conducted using the original data

from these trials. Treatments covered in this section include acetylcysteine, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), creatine, gabapentin, IGF-1, Rilutek®/riluzole, Deprenyl®/selegiline, and thyrotropin-releasing hormone.

The text of this review is followed by two Appendices. Appendix A provides a full citation list of the clinical trials covered in this report. Appendix B includes a summary of each of the 134 clinical trials covered in this report; summaries include the rationale or hypothesis on which each study was based, the intended aim of the study, the primary location of the study, study results, and an overview of major parameters related to study design, patient population, and treatment protocol.

Ultimately, there are two ways of historically interpreting previously published clinical trials in ALS. One arguably misguided interpretation uses contemporary clinical trial design and analytic principles to invalidate the majority of previously published clinical trials. The other interpretation views trial design and therapeutic approaches as necessarily tied to clinical practices and therapeutic paradigms at the time. Under such an interpretation, the ultimate value of a clinical trial lies not in whether it adheres to contemporary design principles, but whether it represented the cutting edge in clinical trial design at the time of its publication, and whether it illuminated issues in design, measurement or analysis that ultimately led to improvements in future clinical investigations.

This report takes the latter of these two approaches, focusing not on the scientific accuracy of each individual trial, but on placing all trials in ALS (and their shortcomings) within a historical narrative in which current clinical trials are part of a process of ever-improving ability to detect or disprove therapeutic effects. Ultimately, this sort of approach will not only help non-specialists in ALS understand the importance of past clinical investigations but will also elucidate the nature and origin of clinical trial design principles that may seem at odds with traditional assumptions about the characteristics of a 'good' clinical trial.

1. Issues in Clinical Trial Design & Conduct in ALS

Before addressing historical trends in clinical trials in ALS, it is important to address the contemporary trial context in which today's clinical researchers assess these earlier efforts at clinical investigation. Both the standards of clinical trials in general and the standards of clinical trials in ALS have changed dramatically over the past forty years. This section will focus on contemporary issues in the design and conduct of clinical investigations aimed at demonstrating the efficacy of a particular treatment in ALS (safety, dose-ranging, and pharmacokinetic studies are a more straightforward endeavor.)

In general clinical practice, the gold standard efficacy study is typically, at the very least, randomized, double-blind, and placebo controlled.[10] Patients are randomly assigned to one of two or more parallel arms of a trial, and then one arm of the study is assigned to receive placebo rather than active treatment. The identity of the medications being taken by each group is kept secret from both doctors and their patients. These design elements are all directed at eliminating different sources of intentional or inadvertent bias in the conduct of these trials.[11]

In the context of this broad standard for clinical trial design, efficacy trials in ALS present their own set of design issues, including the need to account for unusually high variation in rates of progression, the limitations posed by patients' expected post-diagnosis survival time, the difficulty of choosing clinical endpoints in the absence of useful or reliable biomarkers for disease status, concerns over the ability of animal and *in vitro* disease models to predict clinical efficacy, the difficulty of maintaining complete control over patients' treatment regimens, and the challenges of convincing patients to participate in placebo-controlled trials of already FDA-approved or over-the-counter medications.[12]

One of the earliest issues to emerge as a priority for clinical trial design was the wide

variation observed in ALS survival times and rates of progression. (In this context, survival time refers to the time until respiratory failure.)[13] While ALS patients are generally described as surviving an average of 3 years after diagnosis, up to 24% survive for 5 years or more.[14]

On the other hand, between 7% and 10% of patients die in the first year after diagnosis, meaning that a typical efficacy trial that enrolled *only* newly diagnosed patients would already have to plan on losing 7% of the study population during the course of the study. The difficulty lies in figuring out which study participants will have a survival time of 7 months and which will survive 7 years and ensuring that these patients are assigned evenly between the treatment and placebo groups of a trial. Age (≥55 at the time of diagnosis), bulbar onset, rapid time between first symptoms and diagnosis, and early respiratory impairment have all been found to be associated with a shorter survival time.[15] In addition, an array of clinical evaluations can help predict patients' survival time based on their rate of progression.[16] These variables can be used to stratify the randomization process for clinical trials, ensuring the treatment and control groups have an equal distribution of factors that influence prognosis.

However, these clinical evaluations are most accurate at describing time to survival, not the exact rate of progression during each study interval. Despite efforts to develop numerical scales of disease status that decrease linearly over time, the actual course of patients' progression is unlikely to be entirely linear.[17] Thus, while such scales initially promised to reduce the length of clinical trials by providing a surrogate marker of survival, this promise has not necessarily been realized. Clinical investigations still need to collect a range of data points at different intervals in order to develop an accurate linear estimate of disease progression.

Other sources of variation include a sex imbalance in the number of men and women diagnosed with ALS.[18] Although gender does appear to influence one's chances of being diagnosed with ALS (often attributed to hormonal differences between men and women since the difference in diagnostic rates diminishes after menopause), it has not been

reliably demonstrated to play a role in prognosis.

Research has suggested females may have a poorer prognosis than males, but it is unclear whether this is due to differences in the average age of female patients (female patients are more likely to be post-menopausal when diagnosis, and older patients have been shown to have worse prognoses.)[19] Hormonal and genetic factors also appear to cause significant differences in study participants' metabolism of and reaction to therapeutic interventions.[20] In several trials, a distinct portion of the treatment group appeared to respond to treatment while the rest of the treatment group experienced no benefit.[21] In most cases, however, pharmacokinetic studies were unable to identify any variation in drug clearance or bioavailability that could explain this difference.[22]

In certain cases, variation in drug response led researchers to question whether the syndrome currently diagnosed as ALS is actually two or more distinct diseases, although there is no laboratory or epidemiological evidence capable of distinguishing multiple types of ALS, nor are there any relevant etiological or biological differences between familial and sporadic ALS.[23]

Another key issue in clinical trial design has been the selection of appropriate clinical endpoints. In addition to survival, clinical researchers usually take a variety of measurements aimed at assessing the patient's clinical status and, ultimately, his or her rate of decline due to disease. No one measurement has necessarily emerged as the preferred clinical assessment. At least ten different compound scales of clinical progression, each incorporating its own combination of muscle strength, bulbar, respiratory, and activity testing into a final numerical score, have been developed over the past forty years and used in clinical investigations. The most widely used of these scales include the Norris scale,[24] Tufts Quantitative Neuromuscular Exam,[25] Appel ALS Rating Scale,[26] ALS Functional Rating Scale,[27] and Sickness Impact Profile.[28] Although these rating systems have been shown to be highly reproducible from investigator to investigator, the number of rating systems and the failure of clinical trials

in ALS to adhere reliably to one rating system has made it difficult to compare results across trials. Complicating this task is the fact that clinical investigators frequently choose to modify standard scales according to their interpretations of what types of clinical data are most important to track over time.[29] This trend makes it difficult to compare trial results even when the investigators are using the same basic clinical scale.[30]

These efforts at constructing ALS rating scales are in part an attempt to deal with the absence of reliable biological markers of disease. There are no gross anatomical features of ALS that appear in standard clinical imaging techniques (X-ray, MRI, CAT, PET); an absolutely definitive diagnosis still relies on an autopsy, although clinical diagnostic guidelines (known as the El Escorial criteria) have been developed that are reliably predictive of post-mortem findings.[31] Attempts to find a reliable biological or biochemical marker for disease progression have yielded promising results but have so far been unsuccessful.[32]

With no known cause and only one treatment of minimal efficacy, correlating possible biochemical signs of disease progression or regression to clinical observations is difficult. Identifying appropriate biomarkers for ALS would allow pilot efficacy trials to be dramatically shortened and would also permit less labor intensive monitoring of disease progression. Current research into possible biomarkers for ALS will be discussed in the next major section of this report, which deals with current research topics in ALS, but it is important to keep in mind that the absence of appropriate biomarkers for ALS has led to significant issues and inefficiencies in trial design.[33]

A number of other issues in clinical trial design deal with ethical considerations related to patients' expected survival times, placebo-controlled clinical investigation of already FDA-approved or over the counter treatments, and the difficulty of maintaining control over patients' entire treatment regimen during the study. Because patients typically have a limited survival time after diagnosis, this can make them reluctant to participate in placebo-controlled studies in which they may end up receiving placebo for up to 12 or 18 months.[34]

Crossover designs alleviate these fears somewhat by ensuring that all patients receive active treatment for at least part of the study period. Patients are randomized to receive either treatment or placebo to start, and then the groups switch halfway through the study period so that those initially receiving placebo begin active treatment and vice versa. This trial design also has the advantage of requiring fewer patients to demonstrate the same statistical effect as a traditional parallel trial design (in which each group receives either treatment or placebo for the entire study period) but typically must rely on predictors of survival rather than survival rates themselves.[35] Crossover designs were used frequently in the 1980s but have appeared in only a small portion of efficacy trials in recent years.[36]

Most ALS patients are aware that treatments' effects may not always be perceptible, especially for drugs that slow but do not stop progression, and that they may not know whether treatments are effective until the end of the clinical trial. Thus, even in crossover trial designs in which all patients receive treatment at some point, twelve or eighteen months may simply be too long for patients to wait to find out whether the treatment they are taking is futile.

Further complicating this issue is the fact that many investigational treatments in ALS are already FDA-approved for use in other diseases and may be available through off-label prescriptions independent of clinical trials. Certain recent investigational therapies are even available over the counter or from health stores (e.g. creatine, Coenzyme Q10). This means that patients may be reluctant to enter placebo controlled efficacy trials of any sort when they have the option of taking the treatment independently and being assured of not receiving a placebo.

In addition, clinical researchers have few ways of ensuring that patients in one trial do not take additional treatments that may be under investigation in other trials. Depending on the efficacy of these additional drugs and the distribution of their use between treatment and placebo groups, these additional treatments may skew the results of clinical trials or cause unanticipated toxicity. Clinical researchers are thus faced with a range of concerns in addition to basic

statistical and design issues when preparing to conduct clinical trials in ALS.

A number of these issues have been partially resolved through the establishment of consensus guidelines on designing clinical trials in ALS. First developed in 1994, these consensus guidelines were expanded in 1998 and again in 2004 through a conference involving a multinational group of neurologists, statisticians, patient advocates, and representatives from the pharmaceutical industry and an array of regulatory agencies.[37] These guidelines contain a range of recommendations on the appropriate design and conduct of clinical trials in ALS.

However, by their own admission the recommendations describe not the ideal clinical trial, but the absolute minimum required to conduct a valid clinical trial in ALS. Recommendations include specific lengths for safety, pilot efficacy, and full efficacy trials, and specific criteria for enrolling study participants. Particularly noteworthy is the acknowledgement that placebo controls (rather than natural history controls) are still necessary in Phase III efficacy studies. This was an issue of some debate during the late 1990s.[38] A significant portion of the consensus guidelines document is dedicated to a discussion of appropriate statistical planning and analysis. Reviews of earlier clinical trials had highlighted the extent to which 'positive' outcomes were the result

Highlights of Consensus Guidelines

Patient Inclusion
- Between ages of 18 and 85
- Clinical history longer than 5 months
- No more than 5 years post diagnosis
- On no other investigational drugs

Conduct/design
- Phase I minimum 6 months long
- Phase II 6-12 months long
- Phase III needs placebo & sequential design

Information sharing
- Patients should be first to learn about trial results
- Database from study should be available to other researchers after publication

Ethics
- Trials should include an open-label period after controlled study

Statistics
- Careful statistical planning an absolute necessity
- Randomization should be stratified according to covariates highly predictive of outcome
- Distinguish between predictors of progression and effect modifiers
- Post-hoc stratification should be used to generate hypotheses, not confirm them
- Lead-in periods should be used with caution

Endpoints
- Change in muscle strength or survival most useful endpoint
- Surrogate measures of survival, including natural history controls, have yet to be statistically validated
- Quality of life measures should be included in every trial

of faulty or misguided statistical analysis. The guidelines also recommended the use of muscle strength or survival as primary clinical endpoints, and discussed lack of statistical validation of surrogate markers for survival (either biomarkers or clinical rating scales.) Other highlights of the consensus guidelines are outlined in the table on the previous page and should be kept in mind when reading the clinical trial summaries in Appendix B. While it is beyond the scope of this review to evaluate each consensus guideline on a trial by trial basis, the next section traces broad historical trends in ALS clinical trials as they relate to contemporary consensus on clinical trial design in ALS.[39]

2. General trends in clinical trial design in ALS, 1965 - 2004

Although only a limited number of treatments have shown potential benefit in ALS, past failures have also been an occasion for researchers to reevaluate the methods by which they attempted to demonstrate clinical efficacy. The result has been dramatic shifts in the size, design, and scope of clinical trials in ALS. The consensus guidelines for clinical trials in ALS, developed in the late 1990s and early 2000s, are closely tied to these shifts – both to those trends which the consensus guidelines hope will continue and those the guidelines are designed to discourage.

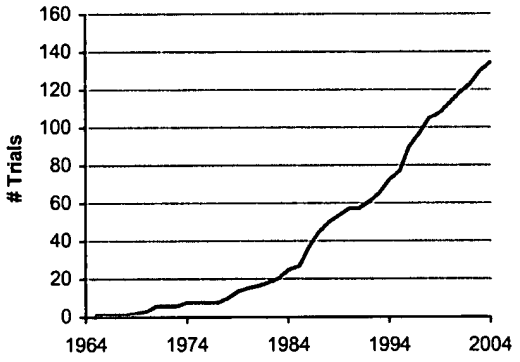
Certainly, trial lengths and the frequency of placebo control in efficacy trials are already well within the range suggested by the consensus guidelines. In a few cases, the current state of clinical trials is at odds with the desires expressed in consensus guidelines – for example, trial population size has increased steadily despite attempts to develop designs that increase the statistical power of small study populations. In some cases, as in the case of sex distribution in trial populations, positive trends in trial design have occurred entirely under the radar of trial guidelines and are not addressed in published recommendations.

This section traces both trends in clinical trial design (as they relate to contemporary issues in trial design) and the types of investigational treatments used in these trials

over the past forty years. It is concerned not with individual treatments or the specific data supporting their use in ALS (these details are covered in later sections), or with assessing the quality of each particular clinical investigation, but instead traces shifting consensus on trial design as expressed through the actual conduct and design of these trials.[40]

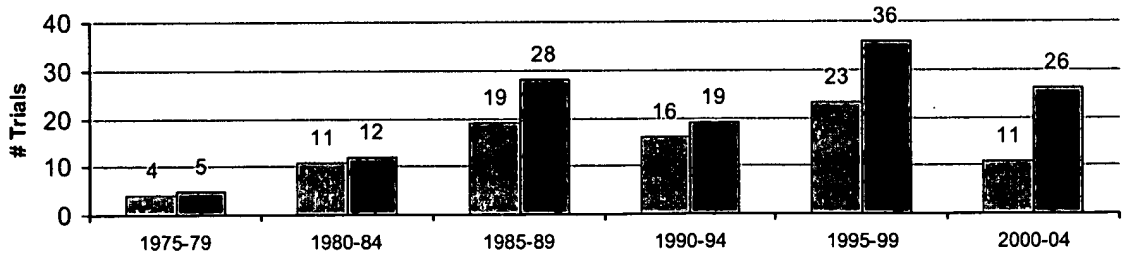
Both the number of published clinical trials and the rate of increase in published clinical trials have risen steadily since 1965. The greatest increases in the number of clinical trials (in terms of the absolute number of new clinical trials published) occurred during the late 1980s and the late 1990s; slightly fewer trials were published between 2000 and 2004 than in the preceding five years. Much of the growth in clinical trials in recent years has been due to an increasing number of safety, dose-ranging, pharmacokinetic, and bio-chemical investigations aimed at developing a better under-standing of drugs' exact biological activity in ALS patients; while in the late 1970s and early 1980s such studies made up less than 20% of all published clinical trials, by the late 1990s they represented a third of these trials, and between 2000 and 2004 this number rose to more than 50% (largely due to ongoing safety and pharmacokinetic studies of Rilutek®.)[41]

Total Clinical Trials in ALS, 1965 - 2004



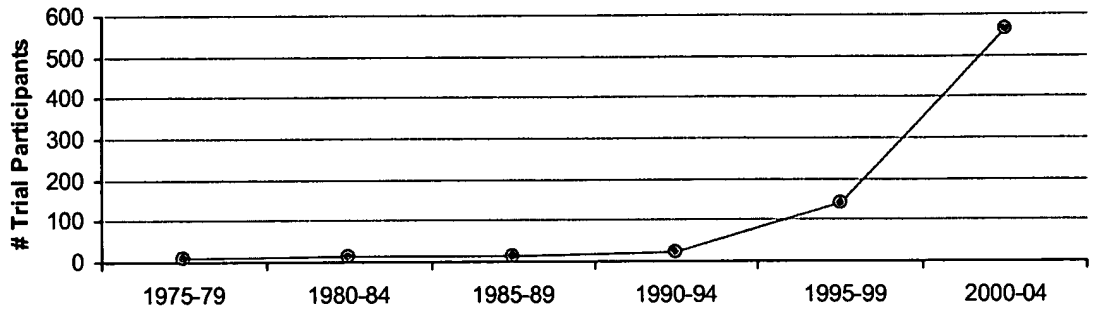
Source: Clinical Trials Database

Total Trials and Trials Directed at Demonstrating Efficacy



Source: Clinical Trials Database

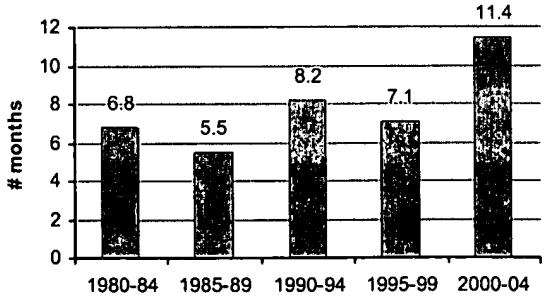
Average Study Size in ALS Clinical Trials



Source: Clinical Trials Database

Despite researchers' interest in designing shorter and smaller trials, the general trend throughout the past forty years has been toward larger and longer trials. From the late 1970s through the early 1990s, the average trial size remained fairly steady, ranging from between 10 and 20 patients on average. In the mid-to-late 1990s, however, average trial size began increasingly rapidly, rising to an average of 140 participants per study between 1995 and 1999 and more than quadrupling to an average of 567 participants between 2000 and 2004.[42] A number of factors contributed to this increase. The 2000 to 2004 period saw the publication of a Phase IIIb safety study of riluzole which enrolled nearly 8,000 participants, which significantly increases the average study size for this period.[43] Even eliminating this outlying data point, clinical trials published between 2000 and 2004 still have an average of 317 participants. The drastic increase in clinical trial size throughout the 1990s and early 2000's can be attributed to three interrelated factors: the organization of multi-center clinical investigation collaboratives on a treatment (e.g. the ALS CNTF Treatment Study Group) and geographic (e.g. the Western ALS Study Group) basis, growing corporate interest in ALS through involvement in clinical trials of Rilutek® (Aventis) and ciliary neurotrophic factor (Regeneron), and the declining popularity of crossover efficacy trials (which require fewer patients.)[44]

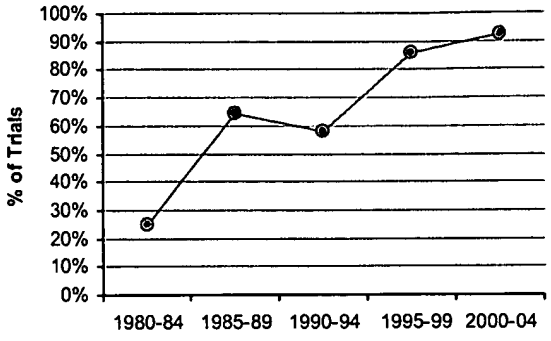
Average Clinical Trial Length in ALS



Source: Clinical Trials Database

In addition to increases in trial size, ALS clinical trials have also increased in length by an average of 4 months since the late 1970s, although for most of that period trial length hovered around 7 months. Although correlating trial length variation to other shifts in trial design is beyond the scope of this review, the increase in trial length between 2000 and 2004 at first appears to be consistent with consensus clinical trial guidelines in ALS which recommended that even safety trials be conducted for a minimum of six months. However, the average safety trial length in the 1980s and 1990s *already* ranged 5 and 6 months; in the 2000 – 2004 period the average safety trial length jumped to 13 months, largely due to a series of Phase IIIb open-label safety trials of Rilutek®.[45] The average length of Phase I safety trials has remained fairly constant and close to the consensus guidelines since at least the early 1980's.[46]

ALS Efficacy Trials with Placebo Controls



Source: Clinical Trials Database

Efficacy trials published between 2000 and 2004 were much more likely to include placebo controls than those conducted in the early 1980s. Although the importance of placebo controls was well documented by the early 1980s, only one out of every four efficacy trials included a placebo arm or time period.[47] In recent years, more than 9 out of every 10 efficacy trials included a placebo group. Placebo controls are particularly important in ALS, since many clinical measures of progression depend on measuring muscle strength (which is highly variable) and patients' subjective assessments of their activities of daily living, both of which can be affected by patients' state of mind. A number of early efficacy trials yielded inconclusive or falsely promising results because the trial design failed to account for the placebo effect.[48]

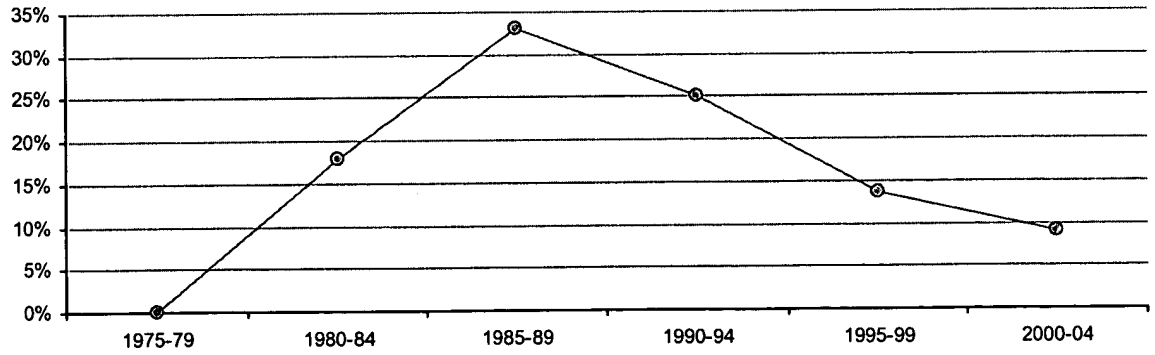
During the 1980s and early 1990s, a number of clinical trials tried to balance the need for placebo controls with the desire to treat all study participants by employing a crossover design. In a crossover trial, patients are

randomized into two or more groups. As discussed earlier, in the typical crossover trial one group starts off receiving the treatment under investigation and the other receives the placebo. At the end of the first treatment period, both groups usually undergo a washout period to allow any residual drug concentrations in the treatment group to be eliminated from the body, and then each group is crossed over to the opposite treatment for a period of time equal to the first.

While this trial design offers significant advantages in terms of study size (and in some cases trial length), it reached its peak in popularity in ALS in the late 1980's and now makes up less than 10% of the efficacy studies published in ALS. The reasons for this decline are not clear, but may have to do with an increasing focus on survival as a more reliably clinical endpoint than muscle strength-based measures of clinical progression. Crossover trials, by design, must rely on these latter endpoints rather than survival, since the decreased study sizes are due to the reductions in variation gained by comparing individual patients' progression during the placebo and treatment periods.[49]

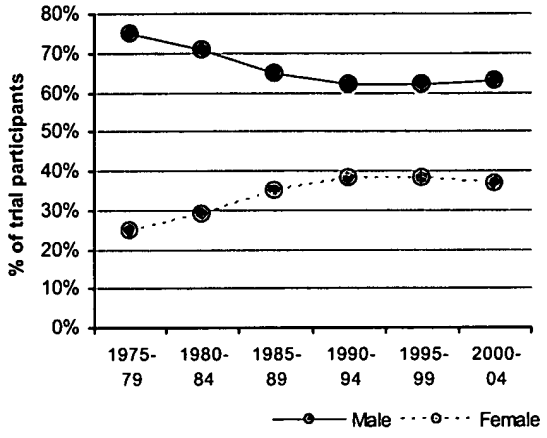
One often overlooked aspect of clinical trials is whether they accurately reflect the sex distribution in the general ALS population. Sex-based differences in drug metabolism are well documented in medical literature, yet until very recently it was still common to conduct clinical trials using only or predominantly male subjects, or to omit analysis of any sex differences in drug

Percent of Efficacy Studies Using Crossover Design



Source: Clinical Trials Database

Gender Balance in ALS Clinical Trials



Source: Clinical Trials Database

response.[50] Including women in disproportionate numbers to the general disease population or excluding women entirely from clinical trials could lead to promising treatments with a sex-specific effect being overlooked *or* being falsely assumed to benefit both men and women despite a predominantly male study population. Complicating the situation is the fact that men are at a higher risk for developing ALS, meaning that the sex distribution in the ALS population differs from the general population. In general, 1.3 to 1.5 men are diagnosed with ALS for every 1 woman, translating to an ALS patient population that is 56% - 60% male and 40% - 43% female.[51] Clinical trials in ALS have approached but not reached this pattern of sex distribution. Between 2000 and 2004, trials enrolled an average of 1.7 men for every 1 woman enrolled, a slightly higher ratio than the highest estimates of sex distribution in ALS. However, this is quite an improvement compared to the late 1970s, when only 25% of study participants were female.[52]

Compared to the earliest clinical investigations, contemporary clinical trials in ALS enroll more participants, last longer, more frequently include placebo controls, and have a more equitable sex distribution among study participants than their predecessors. If the consensus guidelines on ALS become the standard for the design of clinical studies, however, one would hope see a reversal or leveling off of some of these trends. Continued increases in the average trial length or average number of trial participants would certainly be cause for concern given the consensus guidelines' stated desire to reduce trial length and population size through superior statistical planning and design.[53] In addition, if surrogate measures of survival that focus on disease progression rates can be adequately statistically validated, crossover designs may once again rise in popularity. Finally, as researchers refine their

understanding of sex-related or genetic variations in key disease processes, it may be entirely possible that the variation in sex, age, and other characteristics from trial to trial may increase, since study populations may be selected not based on their resemblance to the general disease base, but on their likelihood to respond to the treatment under investigation. These trends, if they occur, should not be interpreted as a 'regression' to outdated modes of clinical trial design, but should be interpreted in the context of the stated goals of clinical trial consensus guidelines, in which trends toward bigger, longer, and more homogeneous studies are not necessarily the ideal direction for clinical trial design in ALS.

3. Etiological Assumptions Underlying Clinical Investigations in ALS

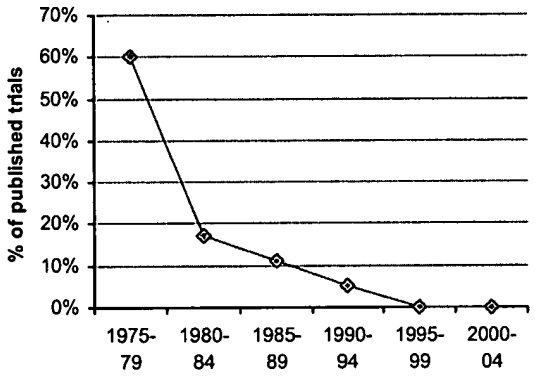
In addition to shifts in clinical trial design, there have been distinct shifts in the reasons investigators initiate clinical trials in ALS. The choice to investigate a particular drug in human subjects must necessarily be based on a strong scientific rationale for believing that drug will be safe and effective in treating the target disease. Usually, the rationale for testing a particular treatment involves not only specific laboratory demonstrations suggesting the possible efficacy of the treatment, but also a statement about the biological causes of the particular disease being studied and the general mechanism of action of the drug under study. In ALS, these statements are necessarily hypotheses, or at best statements about downstream biological effects of the primary disease pathology, since the exact biological cause of the disease is not yet known.

Tracing the rise and fall of these etiological hypotheses, as expressed through the rationale sections of published clinical trials in ALS, provides a window into changes in prevailing scientific thought on ALS over the past forty years. While published pre-clinical investigations over the past forty years have advanced a broad range of etiological hypotheses, not all of these hypotheses were considered equally compelling at the time. Over the past forty years, six major etiological

assumptions about ALS – assumptions that ALS is due to viral infection, autoimmune reaction, neurotrophic deficit, excitotoxic nerve damage, oxidative stress, or metabolic dysfunction – have been used to justify clinical trials, and these assumptions follow distinct patterns of use and disuse over time.

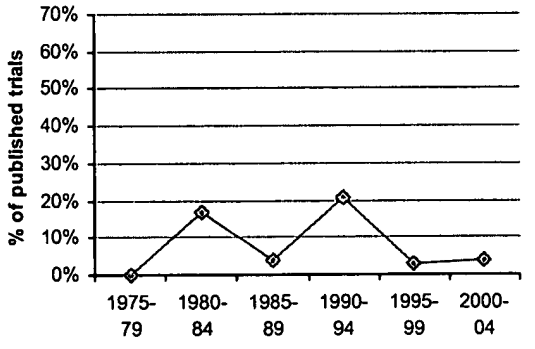
In the late 1970s, the predominant hypothesis behind clinical investigations in ALS was that the disease was caused by a slow acting or chronic viral infection, although trial authors widely acknowledged that there was little laboratory evidence to support this hypothesis. The primary justification for this hypothesis included scattered but irreproducible findings of viruses and virus-like particles in ALS patients pre- and post-mortem, and a growing catalogue of neurological and neurodegenerative ailments which appeared to be caused by chronic viral infections.[54] (A large number of so-called slow virus illnesses that were the focus of intense research interest in the 1970s would later be identified as prion diseases – illnesses caused by infectious protein particles rather than viruses.) Based on this hypothesis, clinicians tested a range of antiviral agents in the hopes of slowing or reversing progression, with little effect. As laboratory evidence in the 1980s began to suggest that earlier findings which had been interpreted as pointing to a viral origin might actually be evidence of an autoimmune component to ALS, the notion of a viral etiology quickly disappeared from ALS trials. While in the late 1970s a viral etiology was used to justify 60% of clinical trials, that number dropped to less than 20% in the early 1980s and

Trials using antiviral agents



Source: Clinical Trials Database

Trials using immune modulators



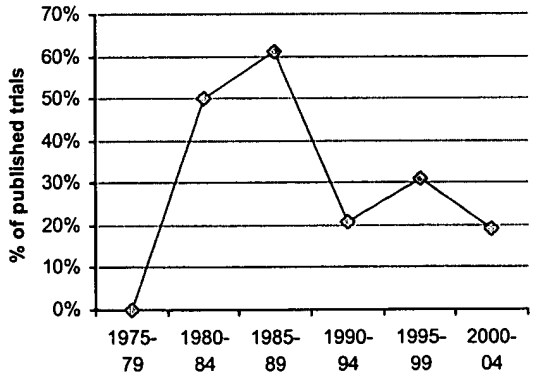
Source: Clinical Trials Database

continued to decline steadily – by 1993, the notion of a viral etiology had completely disappeared from the rationales of published clinical trials.[55]

However, declining interest in a possible viral etiology did not mean that immunological findings in ALS were ignored. Throughout the 1980s and 1990s, trials intermittently focused on investigating treatments and procedures which assumed ALS had an autoimmune component. While findings of viral particles in ALS patients were not easily reproducible, the *signs* of apparent infection were reliably detectible using a range of laboratory techniques.[56] This led researchers to hypothesize that the immune reactions observed were directed not against a rare virus, but against patients' own cells and tissues. Studies based on this etiological hypothesis investigated treatments which had been shown to be successful in treating other autoimmune disorders – primarily plasmapheresis (therapeutic blood plasma exchange) and immunosuppressive drugs and procedures. While these interventions experienced brief popularity in the early 1980s and early 1990s, in recent years they have made up only a small proportion of published clinical trials in ALS.

The early 1980s also saw the sudden emergence of a new etiological hypothesis: the idea that ALS was caused by the body's inability to repair the damage done to neurons by the disease (or, more generally, that the most effective treatment for the loss of motor neurons would be to encourage the body to regrow its dead or dying neurons.) Interest in this particular therapeutic avenue coincided with rapid advances in understanding a range of growth hormones and growth factors, and their potential to yield dramatic therapeutic effects.[57] In terms of sheer number of trials, this etiological hypothesis was most prevalent in the 1980s (particularly the late 1980s, when it appeared

Trials using neurotrophic agents



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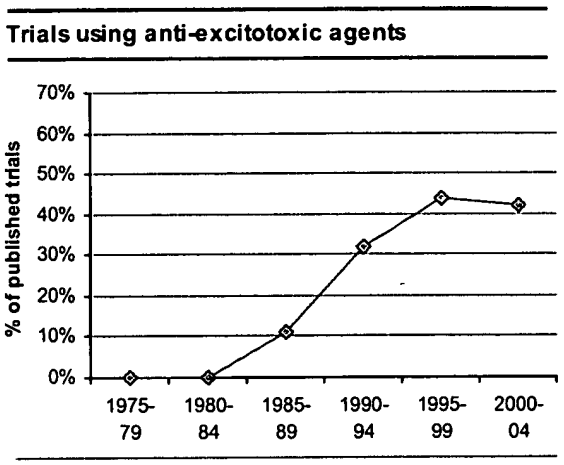

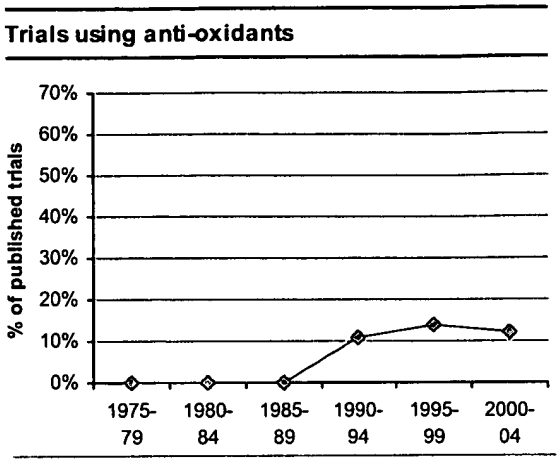

in nearly 60% of published clinical trials.) However, there was a small resurgence of interest in neurotrophic therapies for ALS between 1995 and 1999. Though trials of neurotrophic agents accounted for only 30% of all trials published in ALS during that time period, they were predominantly Phase III efficacy trials. More than 43% of all trial participants between 1995 and 1999 were enrolled in studies testing neurotrophic agents in ALS, even though these studies in absolute numbers represented less than a third of the total clinical published on ALS. The fact that large phase III trials of neurotrophic agents represented only 30% of all clinical trials between 1995 and 1999 was due primarily to the rise of another etiological hypothesis and the accumulation of compelling pre-clinical and clinical data in support thereof. Beginning in the late 1980s, researchers began to conduct clinical investigations which assumed that a key predecessor of motor neuron degeneration in ALS was the flooding of those neurons with massive amounts of synaptic signaling compounds. Usually intended to convey messages from one neuron to the next, when these excitatory compounds accumulate they can be toxic to neurons, a phenomenon called excitotoxicity. Interest in a possible excitotoxic mechanism of action in ALS was based on laboratory findings which suggested animal models of ALS-like syndromes could be induced using excitoxins and data pointing to a possible etiologic role of excitatory amino acids in Huntington's and Alzheimer's Disease.[58] Chief among the possible sources of excitoxicity in ALS was glutamate; studies had shown evidence of altered glutamate metabolism in ALS patients. Strong support

was lent to this hypothesis by investigators' success in demonstrating that Rilutek®, a glutamate antagonist, conferred a small but statistically significant survival benefit to ALS patients treated with the drug. Studies of analogous glutamate antagonists and other anti-excitotoxic compounds have failed to demonstrate similar efficacy, however, and the continued prevalence of excitoxicity as an etiological hypothesis in published clinical trials is due almost entirely to a large number of follow-up safety and efficacy studies on Rilutek®. Even if additional treatments were found to have some efficacy in treating ALS, most researchers assume that excitotoxicity is a downstream event in a much more complicated disease process, and that any therapeutic benefit obtained through anti-excitotoxic treatments will at best have a minimal impact on disease progression.[59]

The 1990s also saw the emergence of oxidative stress as a possible contributing factor to ALS pathology. This interest was due in part to the widespread popularity of research on free radicals, anti-oxidants, and the aging process, as well as a growing interest in anti-oxidants as possible treatments for Alzheimer's and other diseases of aging. A focus on anti-oxidant treatments for ALS was further fueled by researchers' discovery in 1993 that mutations in Cu/Zn Superoxide Dismutase I (SOD1, an antioxidant) were responsible for a significant fraction of familial ALS cases.[60] The mutation has since been identified as a gain-of-function mutation, meaning that familial ALS is caused not by deficiencies in SOD1's ability to mitigate oxidative stress but rather from novel toxic properties possessed by the mutant protein.

However, in the years immediately after the discovery of this gene, a common assumption was that treating patients with either recombinant SOD1 or a range of antioxidants would help slow or reverse the progression of the disease, although this hypothesis has not been borne out in practice. Trials of anti-oxidant treatments have represented between 10 and 15% of clinical trials published in the past fifteen years, but based on disappointing results to date and an evolving understanding of the exact mechanism of the SOD1 mutation, it remains to be seen whether they will continue to appear in such numbers in the future.[61]

In addition to these major trends in etiological assumptions, there have been a range of miscellaneous rationales advanced for trying certain treatments. These rationales have represented between 8% and 40% of clinical trials over time and have included treatments directed primarily at symptomatic management (temporarily improving muscle strength or neural signaling with no expectation of slowing disease progression), studies based on positive therapeutic effects observed in analogous diseases, and studies aimed at confirming anecdotal or case reports of efficacy. In general, no one rationale in this category is prevalent enough to merit a separate discussion at present.

These trends in the rise and fall of certain etiological hypotheses over time help clarify the often confusing array of such hypotheses presented in the introductory paragraphs of many articles on ALS. While the idiopathic nature of ALS means that any number of etiological assumptions might guide clinical research at any given time, there are distinct trends in the prevalence of these hypotheses in the clinical literature over time.

These shifts are not always apparent to readers outside the ALS research community; thus, while a review might offhandedly mention a possible viral etiology among the many theories advanced on the cause of ALS, it is important to place this theory within actual research practice. Though a viral etiology is certainly one of the theories that has been advanced on the cause of ALS, it has long since passed from clinical practice as an

Trends in Etiological Assumptions Behind Clinical Investigations in ALS
(% of total trials conducted in each 5-year period)

	Slow Virus	Autoimmune disorder	Neurotrophic deficit	Excitotoxicity	Oxidative Stress	Misc.
1975-79	60%					40%
1980-84	17%	17%	50%			17%
1985-89	11%	4%	61%	4%		14%
1990-94	5%	21%	21%	32%	11%	11%
1995-99		3%	31%	44%	14%	8%
2000-04		4%	19%	42%	12%	19%

Source: Clinical Trials Database

appropriate target for study. Finally, it is important to keep in mind that the etiological hypotheses advanced in clinical trials usually lag behind pre-clinical and laboratory research by two to four years.[62] As the next chapter (on current research topics in ALS,) will show, a number of promising etiological hypotheses have arisen in the past four years, many of which have yet to appear in published clinical trials but which are being actively investigated both in the laboratory and the clinic.

4. Review of major investigational treatments in ALS, 1965 – 2004

In addition to broad etiological hypotheses, there have been a number of investigational treatments in ALS which have received a great deal of attention. This section reviews some of the major treatments used in published clinical trials on ALS. Treatments included in this portion of the review were selected based on the publication of multiple studies on the same therapeutic compound and on the size of the largest trial conducted (100+ patients for a parallel or sequential trial, 40+ patients for a crossover trial.) These restrictions identified nine investigational treatments which, based on the clinical literature, can be described as receiving the most clinical attention over the past forty years. Each one is reviewed briefly below, and an overview of clinical trials conducted and trials results is included. For a more in-depth description of trial design, rationale, and outcomes, see the appropriate trial summary in Appendix B.

4.1. Thyrotropin-releasing hormone (TRH), a neurotrophic agent

Thyrotropin-releasing hormone (TRH), a tripeptide hormone that stimulates the secretion of thyrotropin from the pituitary gland and also has neurotrophic properties, has almost as lengthy a history of clinical investigation in ALS as Rilutek®, but has been tested only in small scale pilot and crossover efficacy studies. The first such study was published in *Lancet* in 1983 and was based on a hypothesis that the symptoms of amyotrophic lateral sclerosis were primarily due to a metabolic defect.

List of Published Clinical Trials of TRH & TRH analogues

Year	Type	Patients	Length
1983	**Pilot safety & efficacy**	**17**	**<1 month**
1984	Pilot efficacy	6	<1 month
1985	**Pilot efficacy**	**8**	**<1 month**
1986	**Crossover pilot efficacy**	**12**	**<1 month**
1986	Crossover efficacy	41	3 months
1986	Pilot efficacy	30	2 months
1986	Safety/pharmacokinetics	4	6 months
1986	**Pilot efficacy**	**7**	**2-3 months**
1987*	Efficacy	25	<1 month
1987	**Dose-ranging & efficacy**	**19**	**2-3 months**
1987	Pharmacokinetics	15	12 months
1987*	Efficacy	11	2-6 months
1987*	Pilot efficacy	9	<1 month
1988	Efficacy/biomarkers	8	<1 month
1988	Safety	20	5 months
1988	Biomarkers	15	<1 month
1990*	**Pilot safety & efficacy**	**10**	**<1 month**
1990	Biomarkers	6	<1 month
1992	Crossover safety/efficacy	25	6 months

* Indicates study of TRH analogue
Bolding indicates a positive report of efficacy

Only one month long, and conducted in a small patient population with no controls, the promising results of this study would spur eighteen additional studies of TRH and several novel TRH analogues over the next decade, all attempting to confirm or refute these initial findings through a range of study designs, doses, and drug delivery systems. Despite widespread interest in TRH, the largest of these studies (a crossover efficacy study published in 1986) would enroll only 41 patients, and only five out the eighteen studies would report that the treatment was of benefit to study participants. These five reports of efficacy were all based on data from fewer than 20 patients, and consisted of muscle strength or functional improvements in only one or two clinical parameters, such as jaw strength or lower limb strength. In the late 1980s, investigations of analogous compounds and alternative delivery routes (e.g. continuous intrathecal infusion rather than intermittent IV infusion) attempted to reproduce or improve upon these results with little success, and interest in TRH as a possible therapeutic for ALS gradually waned. Later reviews of TRH trials would point out issues in trial design and statistical analysis which called into question the conclusions of these studies.[63]

4.2. Acetylcysteine, an anti-viral agent and anti-oxidant

Acetylcysteine is used as a mucolytic agent to reduce the viscosity of mucous secretions and has also been shown to have anti-viral and anti-oxidant effects. The earliest trials of acetylcysteine were directed primarily toward assessing acetylcysteine's efficacy in alleviating the symptoms of later stages of ALS, where difficulty swallowing and coughing leads to the buildup of excess sputum which in some cases can lead to asphyxiation or respiratory distress. Interest

in acetylcysteine was revived in the mid 1990s in response to a growing interest in anti-oxidant treatment of ALS. In a 12 month, double-blind, placebo controlled efficacy study with 120 participants, treatment with acetylcysteine resulted in a small but not statistically significant survival advantage versus patients treated with placebo. However, this survival advantage was not statistically significant, and the results of this trial have generally been interpreted as failing to demonstrate any benefit of treatment with acetylcysteine.[64]

List of Published Clinical Trials of Acetylcysteine

Year	Type	Patients	Length
1987	Efficacy	40	3-24 months
1987	Efficacy	11	12 months
1995	Efficacy	110	12 months

4.3. Rilutek® / riluzole, an anti-glutamate agent

Rilutek® / riluzole, a glutamate antagonist, is the only FDA-approved treatment for ALS and the only treatment for which clinical trials have repeatedly demonstrated a statistically significant (although small) survival advantage. Originally investigated for a range of neurological conditions, including epilepsy and stroke, by the late 1980s and early 1990s pre-clinical investigations had shown riluzole to be a powerful neuroprotective agent *in vitro*, and had suggested that the source of this neuroprotective effect was the inhibition of both the release and certain post-synaptic effects of glutamate.[65] At the time, research into ALS had begun to suggest that an overproduction or overabundance of glutamate in ALS might play a key role in the biology of the disease.[66]

The first pilot trial, published in 1994, demonstrated a statistically significant survival advantage after 12 months of treatment with riluzole; survival among placebo patients was 58% at the 12-month mark and 74% for patients on active treatment. Among bulbar patients, the difference was more pronounced (35%

List of Published Clinical Trials of Rilutek®

Year	Type	Patients	Length
1994	**Pilot efficacy**	**155**	**12 months**
1996	**Dose-ranging/efficacy**	**959**	**18 months**
1997	**Biomarkers/efficacy**	**5**	**6 months**
1997	Pharmacokinetics	100	1 month
1998	**Reanalysis of trial**	**959**	**18 months**
1998	**Biomarkers/efficacy**	**23**	**<1 month**
1998	Biomarkers	7	<1 month
1999	Biomarkers	17	<1 month
2000	Safety	153	3-36 months
2000	Safety	919	7-8 months
2001	Pharmacokinetics	21	<1 month
2001	Safety	7916	20 months
2002	Safety	168	18 months
2002	Biomarkers	37	18 months
2002	Safety	516	14 months
2002	Safety	2069	3-24 months
2003	Pharmacokinetics	169	<1 month

survival on the placebo and 73% on riluzole.) Although these results did not indicate a dramatic arrest or reversal of progression, they were certainly the largest and most statistically significant demonstration of a survival advantage reported in ALS. Although a larger follow-up efficacy trial was published in 1996, this and subsequent efficacy trials did not demonstrate quite as dramatic a survival advantage. The general consensus upon reviewing all clinical trials of riluzole in ALS is that the drug extends average survival time during the treatment period by 17%, or between 2 and 3 months.[67] Rilutek® was FDA-approved for the treatment of ALS in 1995, but only taken by a fraction of ALS patients due largely to its prohibitive cost and small clinical effect.

However in other countries up to 83% of the ALS population may be treated with Rilutek®.[68] Although the drug's effect may be small, Rilutek offers hope that contemporary therapeutic approaches can gain a foothold in arresting the progression of the disease, and has been credited with eliminating the therapeutically nihilistic attitude which which some physicians previously approached ALS.

4.4. Selegiline hydrochloride / Eldepryl® / Deprenyl®, an MAO-B inhibitor

Selegiline is a selective, irreversible inhibitor of Type B monoamine oxidase with anti-oxidant properties and is used to treat newly diagnosed Parkinson's disease. (Eldepryl® / selegiline and Deprenyl are isomers of the same molecule and have similar biological effects.) Interest in selegiline as a possible treatment for ALS was initially due to its antioxidant properties and its ability to slow Parkinson's disease. Selegiline also appeared to have neuroprotective effects in a rat model of neuronal injury. The treatment has been assessed through three separate efficacy trials, none of which have shown selegiline or Deprenyl® to have any perceptible clinical benefit in ALS.[69]

List of Published Clinical Trials of Selegiline

Year	Type	Patients	Length
1994	Efficacy	111	6 months
1994	Crossover efficacy	10	3 months
1998	Efficacy	104	6 months

4.5. Ciliary neurotrophic factor (CNTF), a neurotrophic agent

Ciliary neurotrophic factor (CNTF) is expressed in glial cells in the central and peripheral nervous systems that appears to be released in response to neuronal injury and

has been shown to have neuroprotective and neurotrophic effects in a range of in vitro and in vivo models of disease.[70] Despite promising results in these in vitro and in vivo studies, two separate efficacy studies of CNTF were unable to demonstrate any clinical benefit of treatment with CNTF. Side effects related to systemic administration of the treatment during early trials may have reduced patients' quality of life; several subsequent trials focused on assessing the safety and tolerability of alternative methods of delivery, including intrathecal pump infusion. While these new delivery methods appeared to be safe and well tolerated, no further efficacy studies of CNTF have been published.[71]

List of Published Clinical Trials of CNTF

Year	Type	Patients	Length
1995	Safety/dose-ranging	57	<1 month
1996	Safety/efficacy	730	9 months
1996	Safety/efficacy	483	6 months
1996	Safety	6	5 months
1996	Safety	72	<1 month
1997	Safety	4	3 months

4.6. Gabapentin, an anti-glutamate agent

Gabapentin, a glutamate antagonist and anti-convulsive agent, was first proposed as a possible treatment for ALS in the mid 1990's due to its neuroprotective effects in an in vitro model of chronic glutamate toxicity and due to early successes in treating ALS with

List of Published Clinical Trials of Gabapentin

Year	Type	Patients	Length
1996	Safety/efficacy	140	6 months
1998	Efficacy	231	9-12 months
2001	Efficacy	128	9 months

Rilutek®, another glutamate antagonist.[72] The first clinical trial of gabapentin in ALS, published in 1996, showed a modest but not statistically significant reduction in rate of progression (as measured by decline in arm strength.) A subsequent trial appeared to demonstrate a statistically significant reduction in the rate of muscle strength decline, but a confirmatory trial failed to replicate this result. On the basis of these inconsistent results, gabapentin is now presumed to have no relevant clinical effect on ALS progression.[73]

4.7. Insulin-like growth factor I (IGF-1), a neurotrophic agent

Insulin-like growth factor-I (IGF-I) is a protein growth factor with widely demonstrated neurotrophic and neuroprotective effects, and was proposed for use in amyotrophic lateral sclerosis based on an array of promising results in *in vitro* and *in vivo* models of diseases related to or relevant to ALS.[74] While the first two trials of IGF-I appeared to

demonstrate statistically significant reductions in the rate of FVC decline and increases in survival time, a third trial was unable to replicate these results.[75] A two-year, double-blind, placebo-controlled study is currently underway at the Mayo Medical Center in Rochester, Minnesota to attempt to replicate the findings of the first two trials and definitively determine whether IGF-I slows progression of weakness in ALS.[76]

List of Published Clinical Trials of IGF-I

Year	Type	Patients	Length
1996	Efficacy	141	9 months
1997	Safety / efficacy	266	9 months
1998	Safety / efficacy	96	9 months

4.8. Brain-derived neurotrophic factor (BDNF), a neurotrophic agent

Brain-derived neurotrophic factor (BDNF), like CNTF, is a protein that has neurotrophic and neuroprotective effects on the central nervous system and brain.[77] Its use in ALS was based on positive results in animal models of Alzheimer's and Parkinson's disease, and on positive preliminary clinical results in other diseases. The first study of BDNF failed to demonstrate a statistically significant survival benefit, but post-hoc stratification suggested that both FVC scores of 91% or lower and adverse reactions to

List of Published Clinical Trials of BDNF

Year	Type	Patients	Length
1999	Efficacy	1135	9 months
2000	Safety / dose-ranging	25	3 months
2003	Biomarkers / efficacy	11	<1 month

BDNF within the first two weeks of treatment were predictive of statistically significant survival advantages on treatment with BDNF. A smaller follow-up study published in 2003 was unable to replicate this survival advantage. A 300- patient study testing the effectiveness of intrathecal and subcutaneous BDNF was terminated in early 2001 after preliminary results showed the trial was highly unlikely to demonstrate an improvement in survival.[78]

4Creatine, a dietary supplement and muscle strength enhancer

Creatine, an amino acid available as an over-the-counter dietary supplement, is currently of interest in ALS primarily for its role in mitochondrial energy production. Studies have shown that mitochondrial dysfunction occurs relatively early in the course of the disease, and the positive effect of creatine on a murine model of ALS suggested to researchers that mitochondria might be an important target for treatment.

List of Published Clinical Trials of Creatine

Year	Type	Patients	Length
2001	Pilot efficacy	28	6 months
2002	Pilot efficacy	27	4 months
2003	Efficacy	175	16 months
2004	Efficacy	104	6 months

The first two published trials of creatine in ALS, however, were primary interested in creatine's effect on muscle strength (creatine is frequently used as a nutritional supplement to help improve muscle strength.) The results of these trials were inconclusive - one suggested that creatine temporarily increases muscle strength in ALS patients, while the other could demonstrate no beneficial effect of creatine on respiratory function. The two most recently published trials focused on creatine's involvement in mitochondrial energy production, and attempted – without success - to demonstrate a beneficial effect on survival. A third efficacy trial, a 9-month, multi-site, sequential placebo-controlled study focusing on survival rates and short-term and long-term effects on muscle strength, is currently being coordinated by the Carolinas Neuromuscular/ ALS Center.[79]

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[5] This number refers only to those clinical trials for which the original full text would be widely accessible to American ALS researchers. An initial list of trials was created using the National Library of Medicine's Pubmed database. "Trials" which were actually case studies of one or two patients, studies which were retrospective rather than prospective, or studies which were clearly not part of a research effort whose end goal was an assessment of efficacy in ALS were obviously excluded. Clinical trials published in languages other than English, German or French were thus excluded from this study. Studies published in scientific journals available in fewer than 10 libraries nationwide were also excluded as unlikely to be read by all but a very small fraction of the ALS research community (roughly 7 studies were excluded using this criteria.)

[6] In absolute numbers, the results 13 ALS and 7 Huntington's disease clinical trials were published between 1964 and 1979. Between 1995 and 1999, the results of 36 ALS and 11 Huntington's disease clinical trials were published. The rate of publication in ALS appeared to decrease slightly between 2000 and 2004 but this may be due to delays in indexing of certain periodicals for 2004.

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[8] The purpose of this report is obviously not to review past trials with a scientific eye or with the intent to discover some new kernel of knowledge on the mechanism of action of riluzole, or whether thyrotropin-releasing hormone is or is not effective in ALS. Scientific reviews of past trials that aim to make definitive statements about efficacy or therapeutic potential are widespread in the literature and will be cited throughout this report as appropriate.

[9] For the purposes of this study, a major efficacy trial is defined as either a traditional parallel 2+ arm placebo-controlled trial with 100+ participants or a crossover trial with 40+ participants.

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[42] Trend lines in the graphs illustrating this and other changes in clinical trial design are included only to better illustrate changes over time and should not be interpreted to represent any statistical modeling or significance assessment of shifts in clinical trial design over time. Standard deviation calculations for all averages have been completed but hold little significance to the content of this report and have not been included or indicated on the graphs.

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Olarte MR, Schoenfeldt RS, McKiernan G, Rowland LP.

Plasmapheresis in amyotrophic lateral sclerosis

Annals of Neurology, 1980, 8(6): 644 - 5.

Hypothesis/Rationale: The authors hoped to establish whether or not circulating factors play a role in amyotrophic lateral sclerosis by testing the effect of plasmapheresis on disease progression.The study was based on earlier studies that showed plasmapheresis was effective in treating myasthenia gravis. The rationale for extrapolating a possible therapeutic effect for amyotrophic lateral sclerosis was based on studies that suggested a possible immune component in ALS, including studies that showed deposits of immune complexes in renal glomeruli, excessive serum complement consumption, and toxic effects of ALS patients' sera on cultured neurons.

Location of Study: New York, NY

Study Results: **No benefit.**

The authors observed no long-range benefit from treatment, nor were any clinical changes - positive or negative - observed immediately after individual treatments.

Study Design	
Length	1 – 6 months
Purpose:	
	Safety
	Dose-ranging
	Pharmacokinetic/molecular
	✓ Efficacy
Controlled?	
Crossover?	
Placebo?	
Randomized?	
Double-blind?	
Blind?	
Phase?	
	✓ Pilot
	Phase I
	Phase II
	Phase III

Study Participant Demographics	
Total Participants	10
Treatment	10
Control	0
Data collected	Norris score

Dosing	
Treatment(s)	Plasmapheresis
Dose(s)/Schedule	2 liters plasma exchange with frequency ranging from 4 treatments in 7 days to 15 treatments over 180 days.

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Kelemen J, Hedlund W, Orlin JB, Berkman EM, Munsat TL.

Plasmapheresis with immunosuppression in amyotrophic lateral sclerosis

Archives of Neurology, 1983, 40(12): 752 - 3.

Hypothesis/Rationale: Attempted to confirm Norris et al.'s 1979 report that plasma removal was effective in ALS, hypothesizing that follow-up trials had failed to confirm this effect because they did not include concomitant immunosuppression.

Location of Study: Boston, MA

Study Results: **No benefit.**
The authors observed no therapeutic benefit of plasmapheresis on the progression of ALS.

Study Design	
Length	6 – 13 months
Purpose:	
✓	Safety
	Dose-ranging
	Pharmacokinetic/molecular
	Efficacy
Controlled?	✓
Crossover?	
Placebo?	
Randomized?	
Double-blind?	
Blind?	
Phase?	
	Pilot
	Phase I
	Phase II
	Phase III

Study Participant Demographics	
Total Participants	8
Treatment	4
Control	4

Dosing	
Treatment(s)	Plasmapheresis, azathroprine
Dose(s)/Schedule	2 mg/kg azathioprine starting one week prior to apheresis procedures. Apheresis (removal of at least 2 liters of plasma during each procedure) occurred three times per week for two weeks, then once per week for three months.

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Bensimon G, Lacomblez L, Meininger V.

A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group.

N Engl J Med, 1994, 330(9): 585 - 91.

Hypothesis/Rationale: The authors conducted a placebo-controlled trial or riluzole to determine whether the anti-glutamate agent was beneficial to patients with amyotrophic lateral sclerosis.Riluzole was found in preclnical studies to modulate glutamatergic transmission, making it an attractive drug candidate to mitigate the glutamate-induced excitotoxicity hypothesized to play a major role in ALS causation.

Location of Study: France

Study Results: **Clear benefit.**

Riluzole conferred a statistically significant survival advantage at the twelve month mark and at the end of the study. After 12 months, the survival rate for placebo patients was 58%, while for riluzole-treated patients it was 74%. Among bulbar patients, the difference was even more pronounced - 35% survival with placebo and 73% with riluzole.

Study Design	
Length	12 months
Purpose:	
	Safety
	Dose-ranging
	Pharmacokinetic/molecular
✓	Efficacy
Controlled?	✓
Crossover?	
Placebo?	✓
Randomized?	✓
Double-blind?	✓
Blind?	
Phase?	
	Pilot
	Phase I
	Phase II
	Phase III

Study Participant Demographics	
Total Participants	155
Treatment	77
Control	78
% Male	58.7%
Data collected	Survival, changes in functional status (using modified Norris score), muscle testing scores, respiratory function, Clinical Global Impression of Change, patients' subjective evaluations.

Dosing	
Treatment(s)	Riluzole
Dose(s)/Schedule	100 mg/day

Drachman DB, Chaudhry V, Cornblath D, Kuncl RW, Pestronk A, Clawson L, Mellits ED, Quaskey S, Quinn T, Calkins A, et al.

Trial of immunosuppression in amyotrophic lateral sclerosis using total lymphoid irradiation.

Annals of Neurology, 1994, 35(2): 142 - 50.

Hypothesis/Rationale: The study explored the possibility that previous attempts to treat ALS failed because suppression was neither powerful enough nor continued long enough to be effective. The authors were attempting both to find an effective treatment for ALS and to probe the specific role, if any, of the immune system in ALS pathology.The study was based on a range of studies which suggested an autoimmune component to ALS, including studies which found circulating immune complexes, increased incidence of a specific histocompatibility type among patients, association with other autoimmune diseases, and calcium-channel-specific antibodies. Although previous trials of immunosuppressive treatments had failed to demonstrate any benefit, the authors interpreted these studies in certain cases to have yielded intriguing and suggestive results inconsistent with a total denial of efficacy.

Location of Study: Baltimore, MD

Study Results: **No benefit.**

The authors found no statistically significant differences in motor function or overall survival. However, total lymphoid irradiation did successfully suppress cellular and humoral immune function throughout the 2-year follow-up period.

Study Design	
Length	23 – 24 months
Purpose:	
	Safety
	Dose-ranging
	Pharmacokinetic/molecular
✓	Efficacy
Controlled?	✓
Crossover?	
Placebo?	✓
Randomized?	✓
Double-blind?	✓
Blind?	
Phase?	
	Pilot
	Phase I
	Phase II
	Phase III

Study Participant Demographics	
Total Participants	61
Treatment	30
Control	31
Data collected	Muscle strength, functional motor activity, humoral/cellular immune status

Dosing	
Treatment(s)	Immunosuppression
Dose(s)/Schedule	Total lymphoid irradiation

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CURRENT RESEARCH TOPICS IN ALS

The previous report, *Clinical Trials in ALS,* discussed the state of clinical research on ALS over the past six decades, tracing the rise and fall of etiological hypotheses and trial design paradigms over time and discussing major issues in the conduct of clinical research in ALS. Clinical investigations, however, provide only a small window of insight into the body of clinically relevant research being conducted in ALS. For one thing, clinical investigations are dependent on the availability and safety of investigational drugs. However, not all potential targets for therapeutic intervention can be modified by known chemical compounds, and not all compounds that affect a particular target are safe or feasible for use in clinical investigations. In addition, the vanguard of published clinical investigations does not necessarily correspond with the vanguard of laboratory research in a disease – conducting clinical research is dependent on obtaining funding, designing a trial, enrolling participants, conducting the trial itself (which may take a year or longer), and preparing trial data for publication. Thus, even the most up-to-date clinical trial data released through conference papers or publication may have been inspired by laboratory research conducted 2, 5, or even 10 years earlier. Understanding the future direction of clinical research in ALS requires close attention to the basic, translational, and pre-clinical research that will ultimately guide future clinical investigations.

Basic and pre-clinical research on ALS has increased dramatically in recent years. More than half of the 8,182 research papers published on ALS between 1945 and mid 2005 were published in the final decade of

that period, and almost a third of all scientific papers on ALS have been published within the past two years.[1] For obvious reasons, the thousands of publications reporting basic and pre-clinical research activities in ALS cannot be reviewed with the same degree of detail as the relatively small number of published clinical studies. Instead, this chapter provides a thorough (but not exhaustive) overview of major contemporary laboratory research topics in ALS that have the potential to impact future clinical therapeutic research. Topics covered include emerging understandings of the biology of ALS, the ongoing search for reliable biochemical markers of disease, the cellular and animal models of disease most frequently used to conduct preclinical studies, and a review of selected emerging etiological hypotheses.

Section I. ALS Clinical Characteristics & Biomarkers

At first glance, clinical perspectives on the basic outward signs and symptoms of ALS appear to have changed little in the past 130 years. Despite drastic changes in diagnostic practices, therapeutic options, palliative care technologies, and understandings of the biological processes behind the symptoms and signs of ALS, textbook descriptions of the core clinical syndrome remain remarkably similar to Charcot's initial definition of ALS – an unusual phenomenon in light of drastic changes in the clinical definitions of other neurological disorders during same time period, particularly multiple sclerosis and Alzheimer's Disease.[2] Yet despite this consistency in defining the traditional clinical signs & symptoms of the syndrome, research in recent years has suggested that the biological impact of ALS may extend beyond motor neurons and the central nervous system.

Elevated levels of glutamate, dysfunction in glutamate uptake mechanisms, increases in oxidative stress, and general alterations in immune system function – all factors believed to contribute to the degeneration of

motor neurons - have all been observed systemically.[3] Other phenomena bear a less direct connection to outward symptoms - researchers have long observed alterations in ALS patients' collagen metabolism, which may help explain the reduced incidence of bedsores among bedridden ALS patients.[4] Additional systemic changes observed in ALS include hypermetabolism and possible sub-clinical involvement of neurons other than the motor neurons, including the autonomic and sympathetic nervous systems.[5]

More importantly, recent studies have suggested a significant overlap between the syndrome of frontotemporal lobar dementia (FTLD) and ALS, with between 1/3 and 1/2 half of all ALS patients also showing signs consistent with FTLD. Traditionally, ALS has been considered a neurological disorder that spares cognition, but the increasing evidence on the overlap between ALS and FTLD challenges this notion. The symptoms of FTLD can be subtle (partial aphasia, behavioral changes) and can easily be overlooked or attributed to the stress of living with ALS by caregivers and patients, which may explain why cognitive involvement was initially ruled out as a component of the clinical syndrome of ALS.[6]

The search for sub-clinical manifestations of ALS and comorbid conditions is relevant to therapeutic research on ALS for several reasons. Many of the etiological hypotheses that have been proposed for ALS deal with more or less systemic phenomena with pathological effects that manifest themselves only in a specific population of neurons. Subclinical pathological changes outside of the spinal cord and central nervous system provide additional pieces of evidence against which to evaluate these etiological hypotheses.

In addition, phenomena external to the central nervous system may provide additional (and possibly more practical) sites for making a definitive diagnosis of ALS. These diagnostic parameters can also be used to assess how accurately animal models mimic human disease.

In addition to elucidating sub-clinical systemic and peripheral changes in ALS, research has increasingly focused on

identifying one or more biochemical markers of disease progression that coincide with or are predictive of observed clinical symptoms. Amyotrophic lateral sclerosis is at a disadvantage compared to many other diseases, because it is difficult to locate, verify, and quantify disease progression in living patients and even more difficult to do so over short periods of time. As discussed in the previous chapter, one effect of these obstacles has been that clinical trials in ALS tend to be quite lengthy, especially given the short survival times of most ALS patients, and must rely on survival rates as the ultimate measure of the clinical effectiveness of a given treatment. In addition, although clear guidelines exist for the diagnosis of ALS through various clinical tests of muscle strength, movement, and reflexes, these diagnostic practices often involve long periods of latency in between observations (in order to check for progression of symptoms) and it may take up to a year or more to verify a diagnosis of ALS.

These characteristics have left clinical practice and research on ALS at a disadvantage compared to other diseases with well established biomarkers, such as prostate cancer and HIV/AIDS. In addition to simply confirming a diagnosis, if ALS does indeed have multiple biological causes which result in the same clinical syndrome, a panel of diagnostic biomarkers could offer the possible ability to distinguish among a range of variant causes of pathology and in so doing determine the appropriate therapeutic intervention.[7]

A more ambitious task envisioned for biochemical markers of ALS pathology is finding a chemical or chemicals whose levels correlate to disease progression. Such markers, at the very least, provide an additional endpoint for clinical trials, and can validate or help standardize clinical observations. Under ideal circumstances, such biomarkers could also be used to significantly reduce the length of clinical trials – even allowing researchers to conduct short screening trials in which changes in key biomarkers after a brief treatment period give an immediate indication of potential therapeutic value.[8]

Unfortunately, the search for biomarkers in ALS has not yet produced a definitive or

widely accepted biomarker or panel of biomarkers for ALS. Although researchers can point to a long list of observed biochemical changes in ALS, these changes have been useful only in distinguishing population-based differences between ALS and other neurodegenerative diseases or healthy controls – not in diagnosing ALS or in improving the accuracy of diagnosis. An overview of these observations, as reported in major review articles on ALS, is provided in Appendix A.[9] Other notable studies in recent years have shown increased levels of the DNA repair enzyme PARP,[10] increased levels of the pro-inflammatory prostaglandin PGE2,[11] increased levels of Substance P,[12] and decreased levels of ICE/Caspase I in the cerebrospinal fluid of ALS patients.[13] Studies of muscle tissues in both ALS patients and mSOD1 mice have also suggested that the differential expression of two isoforms of Nogo, a protein which appears to inhibit neurite outgrowth, may serve as a potential diagnostic marker for ALS.[14]

A number of studies have focused on connecting oxidative stress to disease progression or therapeutic effectiveness; suggesting that the clinical progression of ALS is associated with increases in malondialdehyde,[15] lipid peroxidation,[16] manganese superoxide dismutase (mnSOD) nitration,[17] and total antioxidant status (TAS).[18] However, most of these observations are not unique to ALS, and are likely to be a feature of a range of diseases involving neuronal distress. Apoptosis and peripheral immune system activation have also been proposed as possible areas in which to seek an ALS biomarker, but similar to oxidative stress, the activation of these biochemical pathways are unlikely to be exclusive to ALS.[19] Other notable studies include those showing increased serum levels of APOE,[20] increased serum levels of matrix metalloproteinase MMP-9,[21] and increased serum ICE/Caspase-1,[22] although distinguishing these findings as specific to ALS is a more difficult task.[23]

There have, however, been two promising trends in researchers' biomarker search strategies over the past several years. The first is a growing interest in combining the search for biochemical markers with the adoption of sophisticated neuro-imaging techniques. Unlike multiple sclerosis,

pathological changes in ALS cannot be detected using traditional MRI techniques - the only way to directly measure disease state is through brain or spinal cord biopsies, which for obvious reasons are clinically unfeasible.[24] While lower motor neuron involvement can be fairly reliably determined in the diagnostic process using electromyography, upper motor neuron involvement presents a particular diagnostic dilemma because it traditionally has only been able to be determined using marginally reliable clinical examination techniques that in some cases date back to the late 1800's.[25] In recent years, a number of researchers have turned to proton magnetic resonance spectroscopy as a means of measuring both clinical progression and upper motor neuron involvement.[26] This technique has the advantage of measuring neuronal survival *in vivo* using the ratio of n-acetyl aspartate (a brain metabolite found only in neurons) to creatine as a surrogate marker of neuronal survival – the higher the NAA to creatine ratio, the more neurons surviving in the area analyzed.[27] This approach has been used to validate the observed clinical results of both rilutek and BDNF.[28] It also has diagnostic utility in distinguishing ALS from other neurological diseases.[29]

Although recent studies have cast some doubt on the short term precision of this marker, novel neuroimaging techniques directed at measuring neuronal death over time nevertheless offer a compelling additional surrogate marker of progression for use in clinical investigation.[30]

Another notable trend in the search for biomarkers for ALS is the trend toward searching for 'signatures' – patterns of protein or metabolite expression that can be predictive of ALS diagnosis – rather than one single biomarker. These attempts have taken several forms, from standard blood chemistry studies[31] and HPLC-based studies of specific CSF and plasma metabolite expression[32] to more complicated high-throughput protein fingerprinting techniques.[33] Ramstrom et al. analyzed protein expression in ALS patients' CSF via liquid chromatography and Fourier transform mass spectrometry. They found no specific single biomarker but were able to identify 80% of the ALS patients in the study based on their patterns of protein

expression.[34] Ranganathan et al.'s study of the CSF proteome in ALS patients versus healthy controls, which used surface enhanced laser desorption/ionization mass spectrometry and pattern recognition software, was 92% effective in distinguishing ALS patients from other samples.[35]

However, in most cases these initial protein fingerprinting efforts have been limited to distinguishing between healthy volunteers and ALS patients, while in typical diagnostic practice clinicians must separate authentic cases of ALS from cases of syndromes that mimic ALS. In addition, any utility these protein fingerprints might have is limited to diagnostic purposes, since interpreting changes in hundreds of protein levels is an even more labor intensive approach to measuring disease progression than the 100-item clinical examinations currently used for the same.

It is likely that future investigations into biomarkers in ALS will attempt to overcome these issues. A key question for future investigations is whether diagnostic protein 'fingerprints' can distinguish different variants of ALS (familial vs. sporadic, or even variants within sporadic and familial.) Based on clinical trial, diagnostic, and histopathological data, several researchers have suggested that the causes and downstream events in ALS may be considerably more varied than the relatively uniform outward clinical signs and symptoms. However, aside from data on genetic mutations responsible for ALS, laboratory investigations have not yet been able to distinguish among these purported variants.

Another key question for research is the sensitivity of biomarkers or biomarker panels which track disease progression over time – ideally, researchers hope to find a biomarker or panel of biomarkers that can indicate within a few weeks whether a particular drug has therapeutic potential in ALS. To date, the most promising biomarkers can still only be considered as additional endpoints in standard clinical trials, rather than the primary endpoint in a three-week screening study. Finally, even protein fingerprinting techniques which have successfully distinguished ALS patients from healthy controls face

significant challenges if they are to be transformed from a stand-alone laboratory study into a standard diagnostic test. Recent progress in identifying possible biomarkers and novel biomarker research techniques, in combination with the growth in basic research on ALS, offers hope of overcoming these challenges.

Section II. In Vitro Models in ALS

The establishment of reliable, relevant laboratory models of disease is an important step in investigating both the underlying biology of and possible treatments for diseases. In ALS, the earliest such models were *in vitro* cell culture models, typically in which motor neurons (or cells with similar properties) were subjected to a specific chemical, viral, or bacterial insult which led to their death. As a primarily sporadic disease of idiopathic origin, creating these models posed a slight problem in that rather than infecting cells with a specific pathogenic agent (as done in infectious diseases), researchers had to model the effects of the unknown pathogenic agent using other agents and chemicals, even though testing treatments in a model which mimicked the downstream effects of the disease might have little relevance to alleviating the ultimate cause of disease. Thus, a great number of *in vitro* studies have been based on models in which excitatory compounds are administered to healthy neurons in toxic doses – a model of disease which, despite rather effectively causing motor neuron death, replicates only a portion of the observed biological phenomena in ALS. The issue of constructing *in vitro* models is made more difficult by the clinical impossibility of creating cultures from the affected cells – obviously, *in vitro* models of ALS must use mouse, rat, or fetal cells and, by virtue of involving neurons, must find a way to induce neurons to divide without losing the properties that make the cell culture a relevant model of disease.[36]

Despite these obstacles, a wide range of assays have been developed for ALS. In the absence of a reliable animal model of disease, *in vitro* models were for a long time the primary disease model for testing

possible treatments, and despite the availability of animal models for ALS, the use of *in vitro* models has increased exponentially in recent years with the onset of high-throughput screening programs. In 1995, the number of drugs tested through *in vitro* assays was estimated at 50 (based on published scientific literature); this number has increased to thousands of drugs in recent years, although exact figures and results have not yet been published. *In vitro* results have also been the laboratory basis for at least 6 clinical trials.[37]

Because of the wide number of *in vitro* cell culture models proposed for ALS, and their only partial replication of disease pathology, it is nearly impossible to point to any one standard cell culture model in ALS. Typically, researchers investigating drugs' effects *in vitro* have used a variety of assays – not all of which are necessarily ALS-specific – in order to identify drugs which have high level of activity against a range of processes known to cause or exacerbate neurodegeneration. In general, assays which operate by exposing neurons to excitotoxic compounds or which induce cells to express mSOD1 are accepted approaches to disease modeling, although the details of the cell cultures themselves – the selection of the cell population, means of delivering the toxic substance, and so on – varies significantly from model to model.

The best barometer to date of the 'standard' accepted *in vitro* models are the assays selected for inclusion in NINDS neurodegenerative screening program in which 27 different laboratories screened 1,046 possible neuroprotective compounds in a range of assays related to Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, and ALS. Eleven assays out of the 29 conducted were viewed as relevant to ALS – four relied on mSOD1 to replicate disase, four relied on excitotoxic compounds, and three focused on inducing mitochondrially-mediated apoptosis as a means of inducing neuronal death.[38]

While the earliest in vitro models focused on a single type of cell (usually motor neurons or neurons that mimicked the behavior of motor neurons in some way), over the past several decades, research has gradually uncovered the fact that motor neurons may not be only cell type involved

in ALS pathogenesis. A recent trend in *in vitro* models of ALS has been an increasing focus on organotypic co-cultures of motor neurons and supporting cells (astrocytes, microglia, or both.) Zhao et al., for example, studied both co-cultures of human microglia & motor neurons and microglia, motor neurons, and astrocytes, to trace the role of microglia in initiating motor neuronal injury. [39] In another recent study, Cassina et al. exposed spinal cord astrocytes to fibroblast growth-factor I (FGF-1, which when expressed in astrocytes may play an indirect role in inducing neuronal death) and then cultured embryonic motor neurons on top of the pretreated astrocytes in order to probe the role of reactive astrocytes in inducing motor neuron apoptosis.[40] Other studies have even attempted to preserve the specific environment in which these cells exist by culturing slices of spinal cord.[41] Appendix B summarizes the most recently used *in vitro* models in the six months prior to the publication of this paper. These new organotypic cultures are considerably more complex than the earliest *in vitro* models of ALS, and this added complexity may help to better model the complex disease processes occurring *in vivo*.

Section III. In Vivo Models of ALS

In vivo models, in which diseases are modeled in complete organisms rather than in cultured cells, provide a much more holistic approach to modeling disease compared to *in vitro* models. Animal models of disease have the potential to provide insight not only on the possible therapeutic value of certain drugs, but also on their potential systemic side effects and on the biology underlying the disease being studied. This latter point is especially true for neurological disorders, since the nature of the central nervous system makes it nearly impossible to examine pathological changes in a live patient (in the absence of animal models, researchers must rely on post-mortem pathology findings for their knowledge on disease processes – a process which is biased toward revealing end-stage

disease pathology and obscuring earlier disease events.)

The earliest *in vivo* models for neurodegenerative diseases like ALS were general neuronal injury models created by injuring or administering a neurotoxin to the animal being studied, and hereditary models in which animals expressed naturally occurring mutations that caused ALS-like symptoms. As the genetic basis of familial ALS became better understood in the early 1990s, a number of transgenic models of ALS were created and quickly became the standard for pre-clinical investigations. Recent years have seen the introduction of several 'knockout' models of ALS (models in which a gene is deleted or under-expressed) and the introduction of invertebrate models of ALS which, while asymptomatic, nevertheless may provide a means of creating truly high-throughput *in vivo* drug screening programs. Important questions still remain regarding the exact correlation between the most popular animal model of ALS (the SOD1 mouse) and the human version of the disease. However, the general consensus is that the animal models developed in the past decade represent a powerful research tool in ALS, and that emerging data on possible differences between animal and human manifestations of familial ALS add to rather than detract from the usefulness of these models.[42]

This section reviews the major animal models of ALS, focusing on mouse models of disease. Although bovine, equine, and canine models of ALS exist, most larger animal models of ALS are irrelevant to or impractical for laboratory and pre-clinical research.[43] It is important to note that even with respect to murine models of disease this is by no means a comprehensive account – by 1990 alone, researchers in ALS could point to nearly 40 different animal models which had been proposed for ALS.[44] The discovery of several genes associated with familial ALS in the 1990s, and the subsequent creation of animal models based on those genes, has only increased that number.

However, many of the models that have been proposed for ALS fall far short of modeling human ALS pathology and as a result have not been widely adopted by the

research community. Instead, this section focuses on the most widely used naturally occurring, transgenic, and knockout mouse models of ALS, paying specific attention to the mSOD1 transgenic mouse. (This descriptor refers not to a single mouse strain, but rather to a range of mice expressing different mutant human SOD1 genes at different levels - variations in mutation and copy number result in a wide range of variations in disease phenotype.) The concluding pages of this section cover invertebrate animal models of ALS and general issues related to interpreting the clinical relevance of studies conducted on animal models of ALS.

Naturally occurring rodent models

A number of naturally occurring mutations have been shown to cause ALS-like syndromes in mice. One of the most widely used naturally occurring animal models of ALS – especially prior to the discovering of the involvement of SOD1 in familial ALS - is the Wobbler mouse, which had been identified in the mid-twentieth century but first appeared in the literature on ALS in the early 1980s.[45] Technically, the Wobbler mouse does not have ALS – instead, it expresses a phenotype that has been compared to both progressive muscular atrophy (a variant of ALS affecting primarily lower motor neurons) and Werdnig-Hoffman disease (a form of hereditary infantile spinal muscular atrophy.)[46]

Despite these differences, the Wobbler mouse was initially used to probe the possible causes of ALS – when research shifted to focus on excitotoxicity, however, studies of Wobbler mice revealed significant differences in the types of amino acid abnormalities observed in the brains of Wobbler mice and ALS patients.[47] In addition, the type of glutamate dysfunction observed in the Wobbler mouse was different from that observed in ALS patients – researchers found an increase, rather than a reduction, in NMDA and kainite binding sites in the spinal cord of Wobbler mice.[48] These differences suggested that the neurodegenerative phenotype in Wobbler mice had a different origin than that expressed in human ALS. Later studies have revealed additional pathological changes that support the use of the Wobbler mouse as a model for ALS, including

evidence of cerebral pathology and mitochondrial respiratory chain dysfunction.[49]

Riluzole, thyrotropin-releasing hormone (TRH), insulin-like growth factor I (IGF-I), hyperbaric oxygen therapy, N-acetyl-L-cysteine, various steroids, 7-nitroindazole, leukaemia inhibitory factor (LIF), and SOD1 supplementation have all been tested using Wobbler mice, often with significant benefit. The Wobbler mouse's positive response to a wide range of treatments that have proved futile in clinical trials has cast doubt on its appropriateness as a model for ALS.[50]

Although the Wobbler mouse is arguably the most widely used naturally occurring animal model of ALS, there are several other mutations which can cause ALS-like symptoms. The progressive motor neuronopathy (pmn) mouse, for example, was introduced in the early 1990s and, like the Wobbler, expresses a syndrome that is similar to but not an exact match with human ALS.[51] The pmn mouse has been used in preclinical investigations of several drugs, including GDNF, CNTF, riluzole, and xaliproden.[52] Another naturally occurring model, the MND mouse, expresses a mutation on chromosome 8 which causes late-onset, progressive degeneration of upper and lower motor neurons.[53] The MND mouse initially appearing to be a promising model for ALS, but has been also shown to have additional pathology outside that traditionally associated with ALS and may be a more appropriate model for Batten's disease.[54]

The legs at odd angles (Loa) mouse has a dominant mutation in dynein, a protein responsible for intracellular transport in neurons, and expresses progressive motor dysfunction with age.[55] The Han-Wistar spastic rat has also been used as animal model to study upper motor neuron degeneration in ALS.[56]

Naturally occurring models of ALS can present both an advantage and a disadvantage to pre-clinical research. It is possible that transgenic models of ALS, which are based on relatively rare human mutations, are too restrictive a pathology and inadvertently cause researchers to identify as 'promising' those treatments which have the greatest relevance to less than 2% of all ALS patients. In this case,

naturally occurring models can serve as additional models in which to test possible treatments and a means of ensuring that treatments are acting on biological phenomena common to all forms of ALS. On the other hand, naturally occurring models might produce red herrings in the search for a treatment for ALS – the combination of murine biology and the novel chromosomal location of the mutant gene might combine to create a causative chain that bears little relation to anything encountered in human patients, and which hinders attempts to connect preclinical results to expected results in humans.[57]

Transgenic & knockout rodent models

Transgenic models of disease provide a promising means of overcoming the limitations of naturally occurring disease models. In transgenic models, the observed disease phenotype is caused by the same gene or genes that cause disease in human patients. Despite crucial genetic, physiological, and immune system differences between mice & humans, the mutated genes that cause diseases in people will frequently cause same diseases in animals. Transgenic technology also offers the possibility of accelerating the onset date of late-onset diseases – by inserting multiple copies of a disease-causing gene, researchers can significantly reduce the time it takes for that mutation to cause disease and increase the pace of research.

The first transgenic mouse model for ALS was created through over-expression of the human neurofilament heavy (NF-H) subunit.[58] The observed phenotype in this mouse bears many similarities to human ALS, and only requires a twofold expression of the human gene.[59] Experiments with over- and under-expressing other human and murine neurofilament subunits have yielded interesting and at times puzzling results. Mice over-expressing human NF-H could be 'rescued' from their disease phenotype by a similar over-expression of human NF-L, suggesting that the *balance* between the two neurofilament subunits – not some pathological property of human NF-H – was responsible for the disease.[60]

Normal (1x) expression of human neurofilament medium (NF-M) subunit also

caused ALS-like symptoms, whether NF-M was expressed in addition to or as a replacement for mouse NF-M.[61] Mutant neurofilament light (NF-L) subunit and NF-L knockout mice also showed motor neuron degeneration.[62] It is clear that the various human neurofilament subunits can play a large role in creating ALS-like symptoms in mice, but it is not as clear whether this is relevant to human ALS. One study suggested that mutations in NF-H may lead to ALS, but a separate study failed to identify any abnormal NF sequences in a sample of 100 familial ALS patients known not to carry mutations in the SOD1 gene.[63]

While neurofilament mice have proven a resource in investigating the possible causes of ALS, they have not become widely used as animal model for therapeutic research – mostly because their phenotype does not exactly mimic human ALS. The transgenic SOD1 mouse, created shortly after discovery that mutations in SOD1 can cause familial ALS, has become the animal model of choice for conducting pre-clinical investigations in ALS.[64] It was immediately clear that the SOD1 mouse mimicked human disease not just in symptoms but in biochemical phenomena, including neurofilament-rich axonal swellings, Lewy body-like NF inclusions, Golgi fragmentation, and mitochondrial dysfunction.[65] Early on, researchers also showed that a mutant form of human SOD1 was necessary to cause disease symptoms – overexpression of wild-type human SOD1 only caused mild, sub-clinical damage to neurons.[66]

The particular mutation in the SOD1 expressed by transgenic mice can have a large impact on the phenotype expressed – as in familial ALS, different mutations can lead to different disease courses. The G37R and L38V mutations, for example, have been associated with an earlier age of onset, while the G37R, G41D, and G93C mutations are associated with longer survival times.[67] In mouse models, the G93A mutation is most widely used.[68] Although G93A is not the most common human mutation, it was one of the first to have clinical progression reported in detail, and has short timeline of disease.[69] Massive loss of functional motor units begins at 47 days of age and precedes by 6 weeks the onset of clinical signs.[70] High copy G93A mice tend to show intra-

cytoplasmic vacuoles (which indicate increased damage to the mitochondria) but these do not appear in low copy mice or in familial ALS patients, suggesting a slightly different etiology in high copy mice. Other mutations include the G86R,[71] G85R (which produces a rapidly progressive degeneration and early behavioral changes),[72] G37R, and H46R.H48Q.[73] Despite the availability of a wide range of SOD1 mutations, more than 65% of all published studies using SOD1 mice use mice expressing the G93A mutation.[74]

In addition to tracking the variation caused by different mutations in SOD1, researchers have also probed the effect of additional transgenic modifications on disease progression. These studies have at times yielded puzzling results. For example, although human wild-type (non-mutant) SOD1 does not cause disease in mice, hWT-SOD1 accelerates disease progression when expressed along with mSOD1(G93A), but has no effect when expressed along with mSOD1(G85R).[75] Even more puzzling, genetic aberrations which have been shown to cause ALS-like symptoms independently may actually produce a less severe disease when expressed simultaneously. Thus, deleting murine NF-L in SOD1(G85R) mice slows disease progression rather than hastening it as expected.[76] Similarly, NF-H over-expression slows progression in SOD1(G37R) mice.[77] Dual expression of the naturally occurring Loa mutation and SOD1(G93A) results in a similarly unexpected alleviation of disease progression and extension of lifespan.[78] These surprising results provide insight into the complexity of the biological processes responsible for ALS, and highlight the promising role transgenic animals may play in elucidating the etiology of ALS.

A number of knock-out mice (mice in which specific genes are deleted or underexpressed) have also proven to have ALS-like symptoms. For example, deletion of the hypoxia-response element of the VEGF promoter induces ALS-like symptoms in mice, an interesting finding which expanded possible roles for VEGF (once considered only as angiogenic factor relevant to cancer.)[79] Most recently, mice under-expressing Alsin, a cytoskeletal protein implicated in a juvenile form of familial ALS, were found to develop

progressive motor dysfunction with age.[80] Knockout models – especially those initiated without any intention of modeling ALS (as in the case of VEGF) – may provide a promising means of discovering new proteins implicated in ALS.

Induced rodent models and invertebrate models of ALS

Though less frequently used in published studies, there are a range of contemporary induced models of ALS in which a virus or toxin is used to mimic disease. For example, mice bred to be genetically susceptible to infection with lactate dehydrogenase-elevating virus (LDV) develop ALS-like symptoms shortly after being exposed to the virus.[81] Another induced model of disease uses cycad toxin to create a disease similar to the ALS-Parkinson's Disease complex (ALS-PDC) observed on Guam.[82] The advantages of induced models include lower costs and greater control over the timing of pre-clinical research (studies on transgenic and naturally occurring disease models are necessarily constrained by the age at which animals begin showing symptoms of disease.) However, because there is little evidence that human ALS is caused by a virus or exogenous toxin (except in the case of ALS-PDC), the relevance of these induced models to clinical research is unclear.

Another means of alleviating some of the time constraints imposed by transgenic and naturally occurring mouse models of disease is the creation of invertebrate transgenic models. Invertebrate models offer the advantages of modeling disease in a whole organism, while also offering a study timeline and volume of model organisms similar to those offered by tissue culture models of disease. So far, SOD1 transgenic C. elegans[83] and Drosophila[84] models have been created. Although neither model demonstrates an ALS-like phenotype, mSOD1 does appear to make these models more susceptible to a range of injuries and may yet prove to be an effective model for high-throughput *in vivo* screening programs.

Despite the wide range of animal models available for ALS, and the degree of knowledge on the correlation between their various phenotypes and human ALS, there

are still some general problems which must be overcome.[85] Although the scientific evidence for the clinical relevance of SOD1 mouse study results is strong, this connection has yet to be borne out by the results of clinical trials. Despite the popularity of the SOD1 mouse, positive results in the mouse have only correlated to positive clinical results in humans on one occasion, and those results came before researchers had a sophisticated understanding of pre-clinical study design considerations related to the SOD1 mouse.[86]

Like human ALS patients, mice expressing mutant human SOD1 transgenes demonstrate a wide variation in disease onset and survival time, and also show sex-specific differences in average onset and progression. The difference between female and male SOD1 mice is great enough that segregating treatment and control groups by sex is likely to produce as great an effect as many of the treatments which have been shown to have benefit in the SOD1(G93A) mouse. As work on animal models proceeds, researchers must decide between replicating the characteristics of the human ALS population (which would require more stringently designed pre-clinical studies using larger numbers of animals) and screening studies in which animal populations are specifically selected to have minimal variation in disease onset and progression in order to enable smaller studies and less complicated statistical analysis.

Section IV. Emerging Theories on Disease Etiology

Crucial to the assessment of existing animal models of disease and the creation of new models is an understanding of the specific biological chain of events that precipitates the outward clinical symptoms of disease. The previous chapter of this report discussed several etiological assumptions underlying clinical trials in ALS, including a virus of unknown origin, a deficit of a motor neuron-specific neurotrophic factor,

autoimmune disease, glutamate excitotoxicity, and oxidative stress. Because of the long timelines that often precede clinical investigation, there are a number of theories relating to disease etiology which are currently being investigated in laboratory and pre-clinical studies, but which in most cases have not yet transitioned into clinical investigation. This section provides a brief overview of several of these emerging etiological hypothesis, addressing in turn research on the possible role of protein aggregation, disrupted axonal transport mechanisms, mitochondrial defects, abnormal cell cycle signaling, and proteosomal dysfunction in pathogenesis of ALS. For each etiological hypothesis, general findings that have implicated that particular pathway are reviewed and an explanation of both the general hypothesis (as it relates to all neurodegenerative diseases) and the specific process by which pathology is localized in motor neurons is discussed when available.

Protein Aggregation

Protein aggregates (toxic clumps of misfolded proteins) are a hallmark of a range of neurodegenerative diseases.[87] While the specific molecular content of these aggregates varies among the various neurodegenerative diseases, most aggregates contain misfolded cytoskeletal elements, including filamentous aggregates of neuronal intermediate filament proteins or inclusions with the microtubule-associated protein tau.[88] The general explanation of protein aggregates' role in disease causation is that they cause disease by disrupting cells' quality control systems.[89] However, in nearly all neurodegenerative diseases cells in which protein aggregates are visible and the specific cells that degenerate do not overlap completely. Thus, researchers believe it is likely that readily observable protein aggregates (in the form of microscopically visible inclusion bodies) are a downstream byproduct of pathogenic events that take place on the level of smaller aggregates or perhaps individual misfolded proteins.[90] In addition, although a wide range of protein aggregates are observed in neurodegenerative diseases, protein aggregation and inclusion bodies are also observed in non-symptomatic subjects; for this reason, researchers stress the

importance of distinguishing toxic, disease-causing aggregates from nontoxic ones.[91]

Ubiquitinated inclusions are the most frequently observed types of proteim aggregates observed in sporadic ALS, but the biochemical basis for the formation and the possible toxicity of these aggregates is still not well understood.[92] One area of increasing focus in understanding protein aggregation has been the study of cellular mechanisms for processing and disposing of these aggregates.[93] While inclusion bodies have been observed in the motor neurons of sporadic ALS patients, the most significant data on protein aggregation in ALS comes from the most common genetic cause of ALS: gain of function mutations in SD1. Mmature mSOD1 is highly stable, but the earliest disulfide-reduced polypeptides in SOD1 assembly pathway are highly destabilized and predisposed to forming toxic protein aggregates.[94] Not only does mutant SOD1 demonstrate a tendency to misfold and aggregate, but mSOD1 appears to be able to attract and recruit other proteins typically found in sporadic ALS inclusions.

In addition, although mSOD1 is expressed systemically in affected patients, the localization of pathology to motor neurons can be explained by the high expression of mSOD1 in these neurons, and by the unique cell cycle status of neurons, which ensures that cell death as a last-resort method of quality control results in the irreversible loss of motor neuronal signaling.

It is important to note that protein aggregation on its own does not constitute a free-standing etiological hypothesis. Protein aggregation is nearly always considered in conjunction with the specific cellular processes that are disrupted by (or whose disruption causes) protein aggregation. The following sections address several cellular processes which have been suggested to be dysfunctional in ALS; the role of protein aggregation either as a cause or byproduct of this dysfunction is discussed in each section.

Axonal transport

Disruptions in axonal transport – the process by which various cellular organelles and cytoskeletal elements are moved across the long, thin chamber that makes up more than 99% of the length of neurons – have

long been observed in amyotrophic lateral sclerosis. As early as 1976, researchers were beginning to uncover impairment in axonal transport in ALS patients, and by 1985 the phenomenon was widely recognized as a key event in the pathogenesis of ALS, either as a proximal cause or a downstream event.[95] Axonal transport is particularly important to the survival of neurons, since nearly all cellular materials necessary to axonal functioning must first be synthesized in neuronal perikarya and then transported into (and eventually out of) the axon itself. The processes by which this transport occurs have historically been divided into fast and slow forms of transport based on the rate at which cargo travels through the cell. Organelles and other membrane-based cellular components are transported via fast axonal transport; cytoskeletal elements are typically transported through slow axonal transport.[96] While the earliest studies on axonal transport in ALS mainly revealed alterations in fast axonal transport (specifically, reductions in anterograde and retrograde fast axonal transport velocity and density), later studies in the late 1980's demonstrated that slow axonal transport is also impaired in ALS.[97]

Axonal dysfunction in ALS is not limited to cytoskeletal behavior – ALS-affected axons show distinct changes in overall appearance versus those in healthy neurons, including both a reduced overall axon caliber (diameter) and, often, the presence of giant axonal swellings filled with cytostructural and axonal transport protein aggregates and various axonal transport cargo.[98] These large axonal swellings have been observed to include massive accumulations of kinesin (one of two major molecular motors responsible for fast axonal transport) and highly phosphorylated neurofilaments (cytoskeletal components that regulate axonal caliber), but appear to only rarely contain cytoplasmic dynein (the other major molecular motor involved in fast axonal transport.)[99]

While predicting the effect of serious disruptions in axonal transport is fairly straightforward, hypotheses on the ultimate cause of these changes in ALS are varied. One hypothesis is that oxidative stress deranges neurofilament phosphorylation and assembly, which in turn affects slow axonal transport and leads to accumulation

of neurofilament components and axonal swelling at the site of these accumulations.[100] Others have hypothesized that the hyperphosphorylation of neurofilament subunits is due to the increases in Cyclin-dependent kinase 5 (CDK5) levels which are often observed in neurodegenerative diseases.[101]

In recent years, a variety of transgenic animal models of motor neuron disease have provided new insights into issues of axonal transport, although not entirely clear that results in them translate into humans with the disease. In addition to mutations in neurofilament subunit [H], kinesin, and dynein all causing motor neuron disease-like symptoms in animal models of disease, transgenic mice expressing mSOD1 – a familial-ALS causing mutation in a gene not directly related to either axonal transport or cytoskeletal proteins – also show characteristic alterations in axonal transport.[102] In the low-copy mSOD1 G93A mouse, researchers detected significant decreases in neurofilament proteins, a reduction in axon caliber, and impairment of fast & slow axonal transport coincidental to the appearance of neurofilamentous aggregates and inclusions.[103] High copy mSOD1 G93A mice, which typically have a much more rapid progression of disease, also show similar changes – although in high copy mice impairment of fast axonal transport is more pronounced than impairment of slow axonal transport.[104]

Transgenic mice expressing mutant SOD1 have also provided researchers with insight into the relevance of axonal transport defects at various points in the timeline of disease pathology. In general, reduced transport of specific slow transport cargoes appears to occur long before observable neurodegeneration.[105] In mice expressing mSOD1 G86R, researchers observed a distinct upregulation of proteins related to fast axonal transport and an early decrease in microtubule-associated proteins up to 5 months before the onset of symptoms – changes which were limited to the spinal cord and did not appear in the brain.[106] Expression of mSOD1 has also been observed to alter the cellular localization of the fast axonal transport molecular motor dynein by attracting and incorporating dynein into mSOD1 aggregates.[107] In mice,

both mHuSOD1 and endogenous mouse SOD1 are transported via slow axonal transport in motor and sensory axons of the sciatic nerve, suggesting that mSOD1 may act locally during transport to damage motor axons.[108]

Despite the insights that murine models have provided on a possible role for axonal transport disruption in the pathology of ALS, sorting out the ultimate cause and relevance of these changes may prove more difficult that first expected. Researchers recently crossed mSOD1 mice with Loa/+ mice (legs at odd angles, a naturally occurring mutation in dynein associated with axonal transport impairment and motor neuron degeneration), expecting that the cross would result in exacerbation of motor neuron disease symptoms. Counterintuitively, mice expressing both mSOD1 and Loa/+ mutations had delayed disease progression and a longer lifespan vs. those expressing only mSOD1. The mice also appeared to have a complete recovery in axonal transport deficits vs. those expressing only mSOD1 or Loa/+.[109]

Mitochondrial defects / mitochondrial permeability transition pore

In recent years, increasing attention has been paid to the possibility that defects in mitochondrial function – specifically inappropriate activation of the mitochondrial permeability transition pore that directs mitochondrially-mediated apoptosis – might play a pathogenic role in ALS.[110] In addition to performing critical roles related to aerobic energy production and intracellular Ca2+ buffering, neuronal mitochondria play a key role in neurodegeneration and in both apoptotic and necrotic cell death.[111] While mitochondrially-induced cell death was initially interpreted to be a downstream event of some other pathological process, recent research has focused on the possibility that mitochondrial defects or dysfunction lead to aberrant apoptotic or necrotic cell death.

Researchers have focused on this latter role and the specific changes that mitochondria undergo when regulating or inducing apoptotic or necrotic cell death – in particular, they have focused on the formation of the mitochondrial permeability

transition pore (mtPTP) – a Ca2+ dependent pore in the inner membrane of the mitochondrion. Formation of the mtPTP triggers the release of calcium and other molecules that interact with regulatory proteins involved in inducing apoptosis, and also disrupts the energy-producing functions of the mitochondrion.[112] The formation of the mtPTP also induces massive swelling of mitochondria, which correlates with microscopic findings on mitochondria in ALS.[113] Recent research has cast some doubt on whether the massive mitochondrial vacuolation observed at the onset of ALS is indicative of classical mitochondrial permeability transition or a new form of vacuolation.[114]

Whether mitochondrial involvement in neuronal apoptosis occurs through 'normal' formation of the mtPTP, through abnormal formation of the same, or through derangements in mitochondrial function that lead to mtPTP-like disruptions in mitochondrial membranes, mitochondrially-mediated cell death has been an increasing focus of therapeutic intervention.

Several theories attempt to link 'normal' mtPTP formation with other biochemical phenomenon in ALS. For example, researchers have theorized a link between copper imbalances and mitochondrial dysfunction through the increased production of mtPTP-inducing reactive oxygen species.[115] Others have shown that disrupting spinal mitochondrial function predisposes motor neurons (but not other neurons) to glutamate-receptor mediated toxicity.[116] Because specific patterns of mitochondrial distribution throughout neurons are critical to both neuronal survival and synaptic function, there may also be a link between disruptions in cellular transport mechanisms and mitochondrial dysfunction.[117]

Cell cycle dysfunction

While some researchers consider phenomena like the formation of the mitochondrial permeability transition pore and other apoptotic signaling to be aberrant processes, it is also possible that apoptotic signaling occurs *appropriately* in reaction to other abnormal cellular occurrences. Researchers in recent years have focused on

the possibility that aberrant cell cycle signaling leads to neuronal apoptosis.

Most cells in the human body go through a process of growth and division governed by a series of proteins known as cyclin-dependent-kinases (CDKs), which play a role in cell growth, division, differentiation, senescence, and apoptosis.[118] Unlike most cells, neurons are typically in a quiescent state in most adults, meaning they do not divide or proliferate. Because of this, almost all CDKs are silenced. However, there is a variety of evidence that motor neurons in ALS are activated to reenter the cell cycle and transition from the G(1) to S phase, a shift which may be either the result or the cause of programmed cell death. The evidence in favor of reentry into the cell cycle includes hyperphosphorylation of retinoblastoma protein, increased levels of cyclin D, and redistribution of E2F-1 into cytoplasm of motor neurons & glia.[119]

It has been suggested that cell cycle signaling may be related to changes in the regulation of Cdk5, the only cyclin-dependent kinase which is not typically silenced in adult neurons. (Cdk5 is essential to the development of neurons and is also crucial to the survival of adult neurons.)[120] Researchers have observed Cdk4-mediated cell cycle signaling at the G1-S checkpoint subsequent to Cdk5 deregulation in SOD1(G37R) mice.[121] While Cdk5 and cycling Cdks have no relation in healthy people, in neurodegenerative diseases they may work together to contribute to neuronal death.[122]

Proteasomal dysfunction

Another area of growing interest in ALS research is the possible involvement of the ubiquitin-proteasome system in disease pathology.[123] The proteasome, a barrel-shaped complex of proteins with an interior chamber that contains several active proteolytic sites, is involved in a range of protein degradation pathways, including the regulation of cell cycle protein levels, the degradation of misfolded proteins, and the processing of proteins for antigen presentation.[124] Ubiquitin, a crucial element of proteasomal function, marks proteins for degradation by the proteasome and has long been observed to be a feature of the types of protein aggregates and inclusion bodies observed in ALS.[125]

There is evidence that proteasomal function may be impaired or aberrant in ALS, a phenomena which might explain the presence of these ubiquitinated inclusion bodies.[126] Cells appear to be highly sensitive to even small changes in proteasomal function. For example, a single mutation in active site of 20S proteasome beta5 subunit caused impairment of chymotrypsin-like activity and hypersensitized cells to oxidative stress, triggering accumulation and aggregation of ubiquitinated proteins, and eventually cell death.[127] The source of proteasomal inhibition in ALS is likely to be motor neuron specific rather than systemic, since treatment with proteasomal inhibitors induced general, rather than motor neuron-specific neurodegeneration in organotypic spinal cord cultures.[128]

In certain cases of familial ALS, mSOD1 may be a direct or indirect cause of this proteasomal inhibition. Impairment of proteasomal function (as measured by chymotrypsin-like activity) is observed in mSOD1 mice quite early in the course of the disease.[129] Studies have yielded conflicting data on whether proteasomal impairment is due to simply a decrease in constitutve proteasome levels or whether it represents an abnormal shift in proteasomal function toward the immunoproteasome.[130] Aggregated SOD1 has been shown in a range of studies to be a byproduct of proteasomal inhibition, but may also be implicated directly in that process.[131] It is possible that mutant SOD1 activates the immunoproteasome or otherwise impairs the 20S component (the core 'barrel' component) of the proteasome.[132] Another possibility is that proteasomal inhibition also leads to decreased degradation of Cdk5, inducing its deregulation and setting in motion a chain of events leading to neuronal death. Of the etiological hypotheses discussed in this section, the role of proteasomal dysfunction in ALS is perhaps the least comprehensively explored to date; further research is needed in order to elucidate the specific biochemical phenomena that lead to proteasomal inhibition in motor neurons and to understand the changes in proteasomal conformation that cause the observed inhibition.

Section V. Conclusion and Policy Considerations

Laboratory research and scientific knowledge on ALS has expanded considerably in recent years, providing hope that clinical or laboratory breakthroughs may be imminent. Expanding clinical understanding of the clinical, sub-clinical, and biological manifestations of ALS – even those which appear to have little direct role in disease pathology – has led researchers to increasingly consider models of disease in which peripheral and systemic phenomena cause pathology that is localized to motor neurons, rather than models in which the disease-causing agent originates only in the affected cells. The search for biological markers of disease in both the CNS and the periphery is also beginning to yield promising results with the shift toward more complex and system-based approaches to measuring changes in protein expression. Researchers have a well-characterized array of *in vitro* and *in vivo* models that in many cases closely mimic human ALS, and are increasingly aware of the study design considerations necessary to conduct and interpret the clinical relevance of research using these models. Transgenic mouse models of ALS have also helped point to novel biological pathways that appear to be involved in ALS pathogenesis but which are often involved too early in the disease process to be observed adequately in humans. In the past four years, researchers' knowledge on the possible role of misfolded proteins and protein aggregates in the pathogenesis of ALS has increased exponentially. This new knowledge base promises to provide a strong basis for selecting and testing investigational drugs in future clinical trials.

The purpose of this report was not only to provide a concise overview of major research trends in ALS – most of which are already highly familiar to members of the ALS research community – but to do so in order to introduce policymakers, lay advocates, and non-specialists to the recent history of research in ALS. In addition to exponential growth in research activities, ALS has also

experienced a recent surge in public awareness through both the efforts of celebrity spokespeople and ALS voluntary organizations, and through somewhat troubling rises in the number of people affected by the disease. The attention now directed toward ALS by state agencies, newly formed voluntary organizations, corporations, and patient advocates has the potential to positively impact clinical and laboratory research on ALS, provided that these efforts take into full consideration the history of research in ALS. Policy and advocacy efforts which interpret ALS's status as a medical mystery to mean that there has been a total lack of medical attention to the disease are likely to engage in efforts that either replicate past research or fail to take full advantage of the sophistication of contemporary research on ALS. This report has attempted to communicate not only the promise of current research on ALS but also the complexity and depth of the laboratory research that has been thus far devoted to attempting to solve the medical mystery that is ALS.

There are a number of considerations which organizations interested in entering into the field of ALS research funding may wish to consider. Despite being populated with researchers who care deeply about finding a cause and cure for ALS, the ALS research community is subject to the same pressures as other research communities –pressure to publish, pressure to claim priority on a new discovery, pressure to compete with other research groups. Because of the relatively small size of the ALS research community, these pressures can lead to fragmentation and can detract from the progress of research. Members of the ALS research and policy community have increasingly sought ways to circumvent these pressures, whether through NIH-funded drug screening consortia or less formal working groups organized on a topical or geographical basis. Organizations creating new programs or funding opportunities in ALS should be careful to assess, with the input of members of the ALS research community, whether these new programs create new opportunities for collaboration rather than new opportunities for competition.

Another consideration is the involvement or creation of incentives for the involvement of corporate and other outside interests in ALS research. Although the impact of corporate interests on academic research has been criticized, ALS presents a unique situation in which the benefits of corporate involvement may far outweigh the costs. Especially with the increasing interest in screening programs, in which large numbers of biologically active compounds are tested for an effect against molecular, cellular, and in vivo models of disease, pharmaceutical corporations offer access to large libraries of investigational drugs.

Though these drugs may take years to reach the clinic, they may yet be able to help researchers better understand the etiology of ALS based on their ability to impact various disease models. The involvement of research organizations focused on diseases other than ALS may also benefit screening programs by helping understand differences and similarities among diseases based on the data from screening drugs in models of multiple diseases.

Ultimately, the future of laboratory research in ALS is directly relevant to issues of public health – both now and in the past. Despite Lou Gehrig's death from ALS in 1941 and the public awareness his struggle raised, in the late 1940's it was ultimately multiple sclerosis – not ALS – that became known as one of the nation's top neurological problems. Multiple sclerosis became the focus of intense research effort - one of the first times that a disease that was neither infectious in origin nor widely prevalent (MS affected less than a quarter million people at the time) became the focus of a major public health effort on the local, state, and national level.

Although Gehrig's death had been expected to spur research on ALS, his name was ultimately used to raise money for multiple sclerosis research - largely because ALS was considered a very rare disease at the time compared to multiple sclerosis. Researchers now know that nearly as many people are diagnosed with ALS each year as with MS. The difference in lifespan after diagnosis, however, leads to drastic differences in the prevalence of

the two diseases - it is possible that if ALS became a chronic, treatable condition, that ALS and MS might affect roughly equal numbers of people. Increased government and nonprofit interest in ALS as a public health issue in recent years, along with the promising state of ALS research, provides hope that the public health movement Lou Gehrig's diagnosis was expected to inspire may yet come to pass.

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